THE JOURNAL OF THE
SOCIETY OF DAIRY TECHNOLOGY
17 DEVONSHIRE STREET, LONDON, W.1. *Tel. Langham* 5059

The Journal is issued in quarterly parts, four parts constituting a volume. The parts are usually issued in January, April, July and October each year.

The Subscription rate to non-members of the Society, payable in advance, is £2. 2*s*. 0*d*. net per volume (post free): single numbers, 13*s*. 0*d*. net. Subscriptions and all business enquiries should be sent to the Secretary, Society of Dairy Technology, 17 Devonshire Street, London, W. 1.

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J. Dairy Res. (1961), **28**, 109

A comparison of the hydrometric and gravimetric methods for the determination of solids-not-fat when applied to the milk of individual cows in a single herd

By R. A. EDWARDS

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(*Received* 1 *June* 1960)

SUMMARY. Five hundred and sixty-nine individual cow milk samples from forty-five cows in a single herd were analyzed for fat by the Gerber method, and solids-not-fat by a standard gravimetric method and by the two commonly used British hydrometric techniques. Three hundred and four of the samples were analyzed also for lactose, protein and ash. Large differences between the total solids values obtained by the hydrometric and gravimetric methods were recorded. The magnitude of the differences from the gravimetric values varied with the season of the year and the stage of lactation. The main factor affecting the differences was the protein content of the milk, and equations for the calculation of total solids content incorporating a protein correction factor are given for use if the protein content be known. The hydrometric methods are considered to be of limited use when applied to milks of individual cows.

Several workers have compared hydrometric and gravimetric methods for determining the solids-not-fat (S.N.F.) content of milk. Boden & Campbell (1942) working with herd bulk milks considered the lactometer method (Richmond, 1894) to agree well with the gravimetric, provided that maximum specific gravity had been attained. They quoted mean differences from the corresponding gravimetric results of $+0{\cdot}05$ and $-0{\cdot}018\,\%$ for two series of experiments. In a comparison of the British Standard method (1937), using the minimum density, with the gravimetric method they showed differences of $-0{\cdot}194\,\%$, S.D. 0·065 and $-0{\cdot}165\,\%$, S.D. 0·083 for the same two series. The Scottish S.N.F. Group (1956–57) referring to 1586 herd bulk samples gave mean deviations from the gravimetric method of $+0{\cdot}13\,\%$, S.D. 0·13 for the lactometer method and $-0{\cdot}12\,\%$, S.D. 0·11 for the B.S. method (1937). Rowland & Wagstaff (1959) found the B.S. method to give values which on average were 0·06 % lower than the corresponding gravimetric values for 2425 herd bulk samples. They gave standard deviations of the individual differences from the gravimetric values of 0·08, 0·095 and 0·084 for three different creameries.

In comparing the lactometer value with the gravimetric value Bartlett, Golding & Wagstaff (1932) gave differences ranging from $+0{\cdot}5$ to $-0{\cdot}4\,\%$ for individual cow samples. Ramchandran, Vennateswara & Basu (1953) also working with individual cows found 40 % of the samples to show differences from the gravimetric results of more than $\pm 0{\cdot}25\,\%$ and discrepancies of up to $\pm 1{\cdot}0\,\%$ were observed. The Scottish

S.N.F. Group (1956–57) quoted mean differences from the gravimetric values for 792 individual cow samples of +0·05% for the lactometer method and −0·14% for the B.S. method. Sharp & Hart (1936) showed lactose to have no appreciable effect on the difference between S.N.F. values determined from specific gravity and gravimetrically. They considered that theoretically protein and ash should have an appreciable effect but that in practice this would not be as serious as one might expect, due to other compensating factors. Waite (1959) working with adjusted milks showed that the ratio of casein content to lactose content was the main factor involved. This was borne out in an examination of forty-six herd bulk samples.

The available information indicates that the differences between the hydrometric and gravimetric methods are greater for individual cow samples than for herd bulked milk. In view of the increasing importance being attached to S.N.F. and the recent recommendation contained in a report on Milk Composition in the United Kingdom (1960) that routine recording of the S.N.F. content of the milk of individual cows should be done, further information concerning these differences and their causes seemed desirable.

EXPERIMENTAL

Five hundred and sixty-nine samples of milk from forty-five Friesian cows were examined over a 2-year period. Each sample was an individual cow sample in which two successive morning and evening samples had been bulked according to yield. Each cow was therefore represented several times at different stages of lactation and some in different lactations. Each sample was analyzed for fat content and for total solids by the Richmond and British Standard methods and by a gravimetric technique. Many of the samples (304) were analyzed also for lactose, crude protein and ash. In addition information was available concerning the time of year at which the samples were taken and the stage of lactation of the cow. All samples were stored overnight at a temperature between 0 and 4 °C.

Analytical methods

Gravimetric determination of total solids involved evaporation of about 1 g of milk and weighing the dried residue, phosphorus pentoxide being used as the desiccant. To test the accuracy of the method twelve such determinations were made on each of three well-mixed samples of milk. The standard error of the mean of duplicates was found to be 0·01697, and it would be expected therefore that 95% of duplicate means would lie within ±0·034% of the true total solids figure. To maintain this level of accuracy duplicates differing by more than 0·07% were rejected and the determinations repeated.

The determination of total solids using the density hydrometer was done according to British Standard 734 (1937). Hydrometers calibrated by the National Physical Laboratory were used. The figures have been corrected to bring them into line with the new formula given in British Standard 734 (1959).

The determination of total solids using the lactometer was done on milk which had attained its maximum specific gravity, at a temperature of 60 °F and with instruments calibrated by the National Physical Laboratory.

Fat was determined by the Gerber method. S.N.F. contents were obtained by subtracting the fat percentage from the percentage of total solids.

Lactose was determined by the method of Hinton & Macara (1927) using a modified zinc ferrocyanide coagulation technique, crude protein by a semi-microkjeldahl technique using a selenium and potassium sulphate catalyst mixture, and ash by ignition of the total solids residue at 400 °C.

RESULTS AND DISCUSSION

The mean fat and S.N.F. figures for the 569 samples examined were 3·86 and 8·76 %.

The bias of the hydrometric methods

The two hydrometric techniques were compared with the gravimetric method by subtracting the S.N.F. figure obtained gravimetrically from that obtained by the method under discussion. This difference is referred to as the bias of that particular method, so the word 'bias' is not here used in its true statistical sense.

The biases were sometimes very large, e.g. a negative bias of over 0·70 % S.N.F. was shown by the B.S. method on two occasions and a positive bias of over 0·70 % S.N.F. for the lactometer method on one occasion. The mean bias for the lactometer method was +0·05 % S.N.F. and that for the B.S. method was −0·06 % S.N.F. These mean bias figures indicate that both the hydrometric techniques are capable of giving accurate estimates of milk S.N.F. A more detailed examination of the results from which the means are derived is, however, disturbing. The standard deviations for the individual biases were 0·176 for the lactometer method and 0·178 for the B.S. method. These are considerably larger than those observed by Rowland & Wagstaff (1959) for herd bulk milks.

Thus when we consider the lactometer method the bias may be expected to be outside the range $+0{\cdot}05 \pm 0{\cdot}35$ once in twenty determinations, i.e. the S.N.F. content by the lactometer method will be outside the range −0·30 to +0·40 of the gravimetric value. Thus a milk from an individual cow with a true S.N.F. content of 8·50 % as determined by the gravimetric technique will be shown to have a value below 8·20 % or above 8·90 % once in twenty determinations made by the lactometer method.

With the B.S. method there is a one-in-twenty chance that the bias will be greater than $-0{\cdot}06 \pm 0{\cdot}36$, i.e. that the S.N.F. content by the B.S. method will differ from that obtained gravimetrically by more than −0·42 to +0·30 %. An individual cow sample therefore with a true S.N.F. content of 8·50 % will show a value below 8·08 % or above 8·80 % once in twenty determinations by the B.S. method.

If the milk of an individual cow were to be graded as good or bad using a S.N.F. figure of 8·50 % as standard then, on the basis of the present results with the lactometer method, the sample would have to show a value below 8·26 % to be safely termed bad and above 8·84 % to be safely termed good, working at a 5 % probability level. For the B.S. method the corresponding figures would be 8·15 and 8·73 %, respectively.

Applying the same standard to the present data would have resulted in twenty-three substandard samples being passed and thirty-four samples that were above

standard being unjustly rejected using the B.S. method. Forty-one substandard samples would have escaped detection and thirteen samples would have been unjustly rejected using the lactometer method.

Effect of season on the bias of the hydrometric methods

Mean monthly bias figures for both hydrometric techniques have been calculated and are shown in Table 1. There was obviously a seasonal effect on the bias figures for both methods. For the B.S. method the bias figures agreed with those of Rowland & Wagstaff (1959) in being small in the early months of the year, but the larger differences occurred slightly earlier. The lactometer method showed large positive differences in the early months and smaller differences during the remainder of the year.

Table 1. *Mean monthly differences (bias) between total solids contents (%) determined by hydrometric and gravimetric methods for milk samples from individual cows*

	B.S. method bias		Lactometer method bias	
Month	Year 1	Year 2	Year 1	Year 2
January	+0·06	−0·09	+0·13	+0·11
February	+0·03	−0·15	+0·24	+0·07
March	−0·02	—	+0·18	—
April	+0·01	—	+0·15	—
May	−0·09	—	+0·06	—
June	−0·14	−0·15	−0·11	0·00
July	—	−0·21	—	+0·02
August	—	−0·27	—	−0·08
September	−0·14	−0·23	−0·04	−0·04
October	−0·09	—	+0·02	—
November	−0·13	—	+0·02	—
December	−0·18	—	−0·03	—

In both methods considerable differences occurred in the bias figures for the months common to the two years. The seasonal effect of the bias is therefore not predictable and the introduction of a correction for this effect is not feasible. It is interesting to note, however, that the B.S. method agreed better with the gravimetric method in the earlier part of the year and the lactometer method in the later part.

Effect of stage of lactation on the bias of the hydrometric methods

Mean values for the bias of the methods for samples from cows at the same stage of lactation were calculated and used to construct Fig. 1. A definite trend was discernible which was very similar for both methods. The lactometer bias became increasingly positive for the first 30 days and then became gradually less so as the lactation proceeded, finally becoming negative. The bias with the B.S. method was negative in the early stages; it then became positive and almost immediately reverted to negative. It became increasingly negative as the lactation progressed. As with season no correction for stage of lactation was considered feasible since the bias for a given cow at a given stage of lactation would be unpredictable.

Effect of chemical composition on the bias of hydrometric methods

As a result of the present investigation full details of the lactose, crude protein, ash and fat contents of 304 samples were available as well as bias figures for the two hydrometric methods. These have been used to assess the effect of compositional changes on the bias figures.

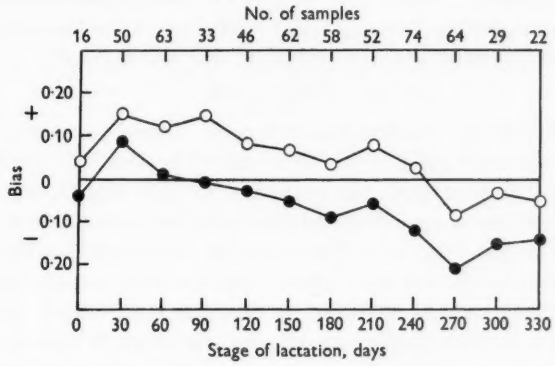


Fig. 1. Effect of stage of lactation on the difference between S.N.F. percentages determined by hydrometric and gravimetric methods. ○, Lactometer method; ●, British Standard method.

Regression relationships were calculated for the biases of both the B.S. and the lactometer methods on the four independent variates fat, protein, lactose and ash. For each method the regression on lactose and ash was non-significant at the 5% level and in the further analysis they were neglected. Regression relationships of the form

$$a+b_1x_1+b_2x_2,$$

where x_1 = % crude protein and x_2 = % fat were then calculated for fat and protein contents for the biases in both the B.S. and lactometer methods. For the lactometer method it was found that

$$b_1 = -0{\cdot}242\ (\pm 0{\cdot}019),$$
$$b_2 = +0{\cdot}028\ (\pm 0{\cdot}014).$$

The value for b_1 was very highly significant, while that for b_2 was just significant at the 5% level, but the actual effect of the fat content on the lactometer method was small and for purposes of prediction may be neglected. The regression coefficient of the bias in the lactometer method on the protein content alone was $-0{\cdot}220\ (\pm 0{\cdot}016)$ and the regression line was given by

$$Y_1 = 0{\cdot}78-0{\cdot}22x_1,$$

where Y_1 = predicted bias of the lactometer method and x_1 = % crude protein.

If we add to the total solids figure determined by the lactometer an amount $0{\cdot}22x-0{\cdot}78$, the difference from the gravimetric result should in most instances be reduced. The standard deviation of an individual difference was estimated to be 0·13 so that for 95% of milk samples from individual cows the difference should not exceed 0·26.

For the bias in the B.S. method the regression coefficients for bias on protein and fat content were found to be

$$b_1 = -0{\cdot}219\ (\pm 0{\cdot}016)$$

and

$$b_2 = -0{\cdot}024\ (\pm 0{\cdot}012),$$

respectively. The value for protein content was again highly significant but that for fat only just so at the 5% level. For purposes of prediction, therefore, fat content can, as before, be neglected. The regression coefficient of the bias on protein content alone was $-0{\cdot}238$ ($\pm 0{\cdot}014$) and the regression line was given by

$$Y_2 = 0{\cdot}68 - 0{\cdot}24x_1,$$

where Y_2 = predicted B.S. method bias and x_1 = % crude protein.

If we add to the total solids figure determined by the B.S. method an amount $0{\cdot}24x_1 - 0{\cdot}68$ the difference from the gravimetric value should usually be reduced. The standard deviation of an individual difference was estimated to be 0·11 so that in 95% of milk samples from individual cows the difference should not exceed 0·22.

It would appear that the ash and lactose contents of the sample had little effect on the magnitude of the bias, as predicted by Sharp & Hart (1936). Both protein and fat contents had a significant effect on bias but variation in protein content was by far the more important. The latter accounted for 39% of the variance in the lactometer method bias and 50% in the B.S. method bias. This is in agreement with the views of Waite (1959) who considered casein to be the main component affecting the bias of the B.S. method. Waite, however, stated that fat content had no effect on bias whereas the present analysis indicates that it does have a significant though small effect.

Correction factors based on the protein content could be introduced into the equations for calculating total solids as follows:

$$\text{total solids \%} = 0{\cdot}25G + 1{\cdot}21F + 0{\cdot}22P - 0{\cdot}64,$$

$$\text{total solids \%} = 0{\cdot}25D + 1{\cdot}21F + 0{\cdot}24P - 0{\cdot}02,$$

where G = lactometer reading at 60 °F; D = density hydrometer reading at 20 °C; P = % crude protein; F = % fat.

CONCLUSION

In the application of hydrometric techniques to the determination of total solids in milk from individual cows, large differences from the gravimetric value occur. The differences are affected by season and stage of lactation, the effect apparently being due to changes in chemical composition. Of the main chemical constituents, lactose and ash do not affect the magnitude of the difference. Fat has a significant, but small, effect. Protein is the factor having the greatest influence on the differences, and the accuracy of the hydrometric methods could be improved by introducing a correction for protein content into the equations for calculating the total solids. If this involved the additional determination of protein it would clearly not be practicable since it would be easier and quicker to determine the total solids gravimetrically, but if the protein content were being determined for other purposes the application of the correction would give some gain in accuracy. The data presented indicate that the hydrometric procedures are not suitable for the accurate determi-

nation of S.N.F. for the milk of individual cows and that even as rough sorting tests for such milk their use is limited.

The author thanks Dr J. N. Lawley for advice concerning the statistical analysis of the data and Miss M. E. Simpson for valuable technical assistance.

REFERENCES

Bartlett, S., Golding, J. & Wagstaff, A. W. (1932). *Agric. Progr.* **9**, 95.
Boden, S. M. & Campbell, C. H. (1942). *J. Dairy Res.* **13**, 45.
British Standards Institution (1937). *B.S.* 734; (1959). B.S. 734.
Hinton, C. L. & Macara, T. (1927). *Analyst*, **52**, 668.
Milk Composition in the United Kingdom (1960). London: H.M.S.O.
Ramchandran, K. S., Vennateswara, Rao R. & Basu, K.P. (1953). *Indian J. Dairy Sci.* **6**, 111.
Richmond, H. D. (1894). *Analyst*, **19**, 73.
Rowland, S. J. & Wagstaff, A. W. (1959). *J. Dairy Res.* **26**, 83.
Scottish Milk Solids-not-fat Group. (1956–57). *3rd Annu. Rep.* Ayr: The Hannah Dairy Research Institute; Edinburgh: The School of Agriculture.
Sharp, P. F. & Hart, R. G. (1936). *J. Dairy Sci.* **19**, 683.
Waite, R. (1959). *Proc. XVth Int. Dairy Congr.* **3**, 1559.

Printed in Great Britain

J. Dairy Res. (1961), **28**, 117
With 1 *plate.*

Physical properties of milk fat.

II. Some factors influencing crystallization

By J. M. de MAN
Department of Dairy Science, University of Alberta, Edmonton, Canada

(*Received* 31 *October* 1960)

Summary. The crystal habit of milk fat is determined by the cooling procedure employed and is changed markedly by interesterification (randomization) of the fat. X-ray diffraction analysis indicated that slowly cooled milk fat occurs in both beta prime and beta modifications, whereas in rapidly cooled milk fat, evidence for the beta prime modification only was obtained. The long spacing was greatly increased by interesterification, especially when the fat was slowly cooled. The presence or absence of certain high melting glyceride fractions greatly influenced solid fat content, softening point and hardness of milk fat.

The crystallization behaviour of milk fat is rather complex, mainly because it contains an exceptionally large variety of fatty acids and glycerides. It is well known that different cooling rates will result in large hardness variations in the fat; this is the cause of excessive hardness in butter made by some continuous processes. Mulder (1953) has explained this effect by assuming that rapid cooling results in the formation of mixed crystals. This is a feasible explanation as mixed crystal formation would occur preferentially in a fat with a large variety of similar glycerides.

Polymorphism has also been suggested as a possible cause for the difference in properties between rapidly and slowly cooled milk fat. Thomas (1950) found evidence of the occurrence of polymorphism in milk fat fractions but not in the whole milk fat. Mulder (1953) has described the occurrence of a 'metastable' form of milk fat. A double melting point was observed by a microscopic method when rapidly cooled cream was heated. The phenomenon could only be observed within a short period after cooling of the cream, and it was assumed, therefore, that the metastable form changed rather rapidly into a more stable form. In the same paper mention is made of the possibility of the formation of a glassy state of butterfat, but no experimental evidence for its existence is available. As the rapid and slow cooling of milk fat causes differences in crystal size (de Man & Wood, 1959) and in solid fat content, as will be shown in this paper, it is very difficult to judge the importance of polymorphism as a cause of the different physical properties of rapidly and slowly cooled milk fat.

During the course of a study on the influence of chemical modification on the physical properties of milk fat, it became apparent that the presence of certain high melting glyceride fractions and the specific glyceride structure of the fat greatly influenced its crystallization behaviour. The research reported here was undertaken

to elucidate the nature of the difference in crystal structures in slowly and rapidly cooled milk fat and to determine the importance of the high melting glycerides in crystallization behaviour and its effect on some physical properties of the solidified milk fat.

EXPERIMENTAL

Polarized light photomicrographs were made by the procedure described previously (de Man & Wood, 1959). X-ray diffraction powder patterns were obtained with a Philips Geiger counter diffractometer. The samples of pure dry milk fat were melted and poured into the cell mounts. Cooling was done in two ways, slow cooling in an air-incubator at 15 °C, and rapid cooling in a freezer at − 20 °C. The samples were left at these temperatures overnight and then tempered at 0 °C. Just before placing the samples in the X-ray beam the surfaces were smoothed with a spatula. During the measurement the temperature of the samples was maintained at about 0 °C by packing the sample holder and shield with dry ice. The radiation used was Cu, Kα (λ = 1·5418 Å); the samples were scanned at the rate of one degree per minute for 2θ angles from 2 to 80 degrees.

Solid fat content, hardness and softening point were determined as described in part I of this series (de Man, 1961).

RESULTS

Rapid cooling of milk fat results in the formation of very small crystals as shown in Pl. 1*a*, whereas slow cooling results in the formation of relatively large crystals (Pl. 1*b*). After interesterification, these characteristic crystal forms are no longer apparent; the crystals in the slowly cooled sample are now smaller than those in the rapidly cooled sample (Pl. 1*c* and *d*).

X-ray diffraction patterns of slowly and rapidly cooled milk fat were recorded, both before and after interesterification. From the patterns the '*d*' spacings were calculated and these together with their relative intensities are listed in Table 1. The more important parts of the diffraction patterns are presented in Fig. 1. All samples were characterized by strong short spacings at 3·8 and 4·2 Å, while the original slowly cooled sample had an additional strong spacing at 4·6 Å. With the

Table 1. *The influence of interesterification and rate of cooling of milk fat on X-ray diffraction patterns*

Original slowly cooled		Original rapidly cooled		Interesterified slowly cooled		Interesterified rapidly cooled	
'*d*' spacing, Å	relative intensity	'*d*' spacing, Å	relative intensity	'*d*' spacing, Å	relative intensity	'*d*' spacing, Å	relative intensity
20·6	3	20·9	3	24·7	3	21·5	2
13·9	7	13·9	11	14·1	8	14·0	12
4·6	38	4·2	79	4·2	88	4·2	80
4·2	74	3·8	45	3·9	3	3·8	43
3·8	36	1·4	2	3·8	51	2·3	5
2·3	2	1·2	4	2·3	2	2·0	10
2·0	2	—	—	2·0	2	1·4	3
1·4	1	—	—	1·4	1	1·2	12
1·2	2	—	—	1·2	1	—	—

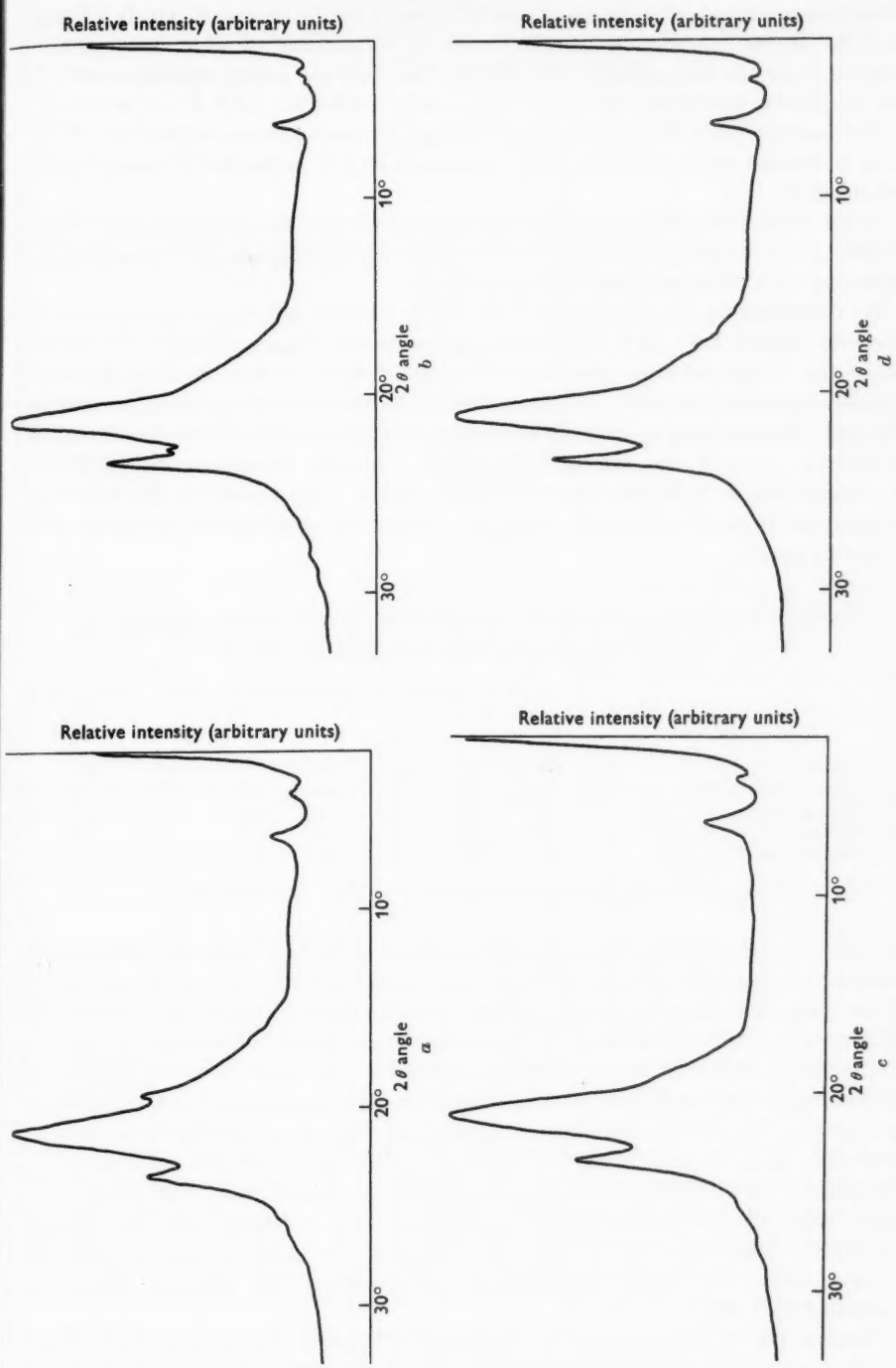


Fig. 1. X-ray diffraction patterns of milk fat covering the range of 2θ angles from 2 to 34°. (*a*) Slowly cooled; (*b*) slowly cooled, interesterified; (*c*) rapidly cooled; (*d*) rapidly cooled, interesterified.

procedure employed '*d*' spacings of over 30 Å could not be measured, but it is likely that the largest spacings measured correspond to one-half of what is commonly referred to as the long spacing. This would then indicate a long spacing of 41·2 Å for the slowly cooled fat, 41·8 Å for the rapidly cooled fat, 49·9 Å for the slowly cooled interesterified fat and 43·0 Å for the rapidly cooled interesterified fat. There is an indication of an additional long spacing at 42·0 Å in the slowly cooled interesterified fat.

As the major effect of interesterification is a large increase in high melting glycerides (HMG), it was decided to determine the role of this glyceride fraction in crystallization behaviour and its effect in influencing hardness.

By dissolving 10 g of September milk fat in 20 ml of ether and adding 5 ml of absolute ethanol, 2·2 % of a high melting glyceride fraction crystallized out at 15 °C. When the volume of ether was reduced to 10 ml, 6·5 % of high melting glyceride fraction crystallized at 15 °C. To some of the September fat 2·2 % of the high melting glyceride fraction first mentioned was added so that a series of fats was obtained as follows: 1. milk fat minus 2·2 % HMG. 2. milk fat minus 6·5 % HMG. 3. normal fat. 4. milk fat with 2·2 % HMG added. With these fats the following measurements were performed: solid fat content by dilatometry, hardness and softening point.

Table 2. *The effect of varying the concentrations of high melting glycerides in milk fat on softening point and solid fat content*

Type of milk fat*	Solid fat content (%) at temperatures (°C) of						Softening point, °C
	10	15	20	25	30	35	
Original	58·0	42·3	21·2	13·7	7·0	1·5	32·6
Original slowly cooled	45·5	33·4	18·5	12·3	7·2	1·7	—
Original minus 2·2 % HMG	56·1	39·2	18·0	9·0	3·1	0	29·7
Original minus 6·5 % HMG	49·7	31·0	9·7	1·8	0	—	24·5
Original plus 2·2 % HMG	69·2	53·5	33·3	25·8	19·9	12·9	35·4

* Standard cooling procedure is rapid, unless otherwise indicated, see text.

Solid fat contents and softening points are listed in Table 2. While the dilatometric measurements are normally run on fat that is cooled in the dilatometers by immersion in ice-water, a series of measurements was made on the original fat after cooling the dilatometers slowly in air at 15 °C. There was a considerably lower solid fat content in the slowly cooled fat at the lower measuring temperatures. Removal of 2·2 % of HMG resulted in lowering of solid fat content at all temperatures but the reduction was greater than 2·2 % at the higher temperatures, whereas at the lower temperatures it became closer to this value. The lowering in softening point amounted to 2·9 °C. Removal of 6·5 % HMG lowered the solid fat contents considerably more than 6·5 %, again relatively more at the higher temperatures. The softening point was reduced by 8·1 °C. Addition of 2·2 % HMG resulted in a very large increase in solid fat content, over 10 % at all measurement temperatures. The softening point was increased by 2·8 °C.

Penetration hardness values are tabulated in Table 3. Removal of 2·2 % HMG resulted in lower hardness in all cases. Removal of 6·5 % HMG decreased the hardness

Table 3. *The effect of rate of cooling and concentration of high melting glycerides on penetration hardness of milk fat*

Type of milk fat	Method of cooling	Penetration hardness (10 g) at temperatures (°C) of		
		10	15	20
Untreated	Slow	103	67	0
Untreated	Rapid	242	132	41
2·2% HMG removed	Slow	77	0	0
2·2% HMG removed	Rapid	197	91	0
6·5% HMG removed	Slow		remained liquid	
6·5% HMG removed	Rapid	130	60	0
2·2% HMG added	Slow	134	89	3
2·2% HMG added	Rapid	528	211	31

even more; the slowly cooled samples failed to solidify under the cooling conditions employed. Addition of 2·2% HMG resulted in increased hardness in all samples but the greatest increase occurred in the rapidly cooled sample at 10°C.

DISCUSSION

The change in crystal size resulting from interesterification has been observed with lard (Hoerr & Waugh, 1955) and with various fat-mixtures (Becker, 1959). The peculiar inversion of crystal size on rapid and slow cooling of original and interesterified milk fat apparently is the result of the change in glyceride composition. This again emphasizes the importance of certain small glyceride fractions in determining crystal growth.

The X-ray diffraction data indicate that in both original and interesterified fats the crystals occur in the beta prime modification as indicated by the strong spacings at 3·8 and 4·2 Å. The slowly cooled original sample also shows a strong spacing at 4·6 Å which is indicative of the beta modification (Lutton, 1948). This would seem to indicate that slow cooling results in crystallization of the fat in both beta prime and beta modifications. The 4·6 Å spacing is completely absent in the rapidly cooled fat and the interesterified fats, again indicating that a specific fraction of the fat is involved. The occurrence of different polymorphic forms in milk fat has not heretofore been unequivocally demonstrated by this method. The long spacings are essentially the same in the original fat when slowly or rapidly cooled, but differ from those of the interesterified samples. An increase in long spacing was found in lard after interesterification (Hoerr & Waugh, 1955) though no information on cooling rates is supplied in this paper.

Solid fat contents are influenced by the presence or absence of the HMG. The data obtained indicate that certain fractions of the milk fat cause closely related but otherwise soluble fractions to co-precipitate when crystallization is initiated. The remarkable increase in solid fat content of the sample with added HMG is a good demonstration of this phenomenon. The softening points are greatly influenced by removal or addition of HMG and this is equally true for hardness. There is as a general rule an increased hardness when solid fat content and softening points increase and decreased hardness when solid fat content and softening point decrease,

but the extent of hardness change cannot be predicted from the magnitude of softening point increase nor from the increase in solid fat content.

The author expresses his gratitude to the Alberta Research Council for making the X-ray diffractometer available and to Dr L. B. Halferdahl for carrying out the X-ray diffraction measurements described in this paper.

REFERENCES

BECKER, E. (1959). *Fette, Seifen, Anstrichmitt.* **61**, 1040.
DE MAN, J. M. & WOOD, F. W. (1959). *Proc. XVth Int. Dairy Congr.* **2**, 1010.
DE MAN, J. M. (1961). *J. Dairy Res.* **28**, 81.
HOERR, C. W. & WAUGH, D. F. (1955). *J. Amer. Oil Chem. Soc.* **32**, 37.
LUTTON, E. S. (1948). *J. Amer. chem. Soc.* **70**, 248.
MULDER, H. (1953). *Neth. Milk Dairy J.* **1**, 149.
THOMAS, A. (1950). Thesis, University of Minnesota.

EXPLANATION OF PLATE 1

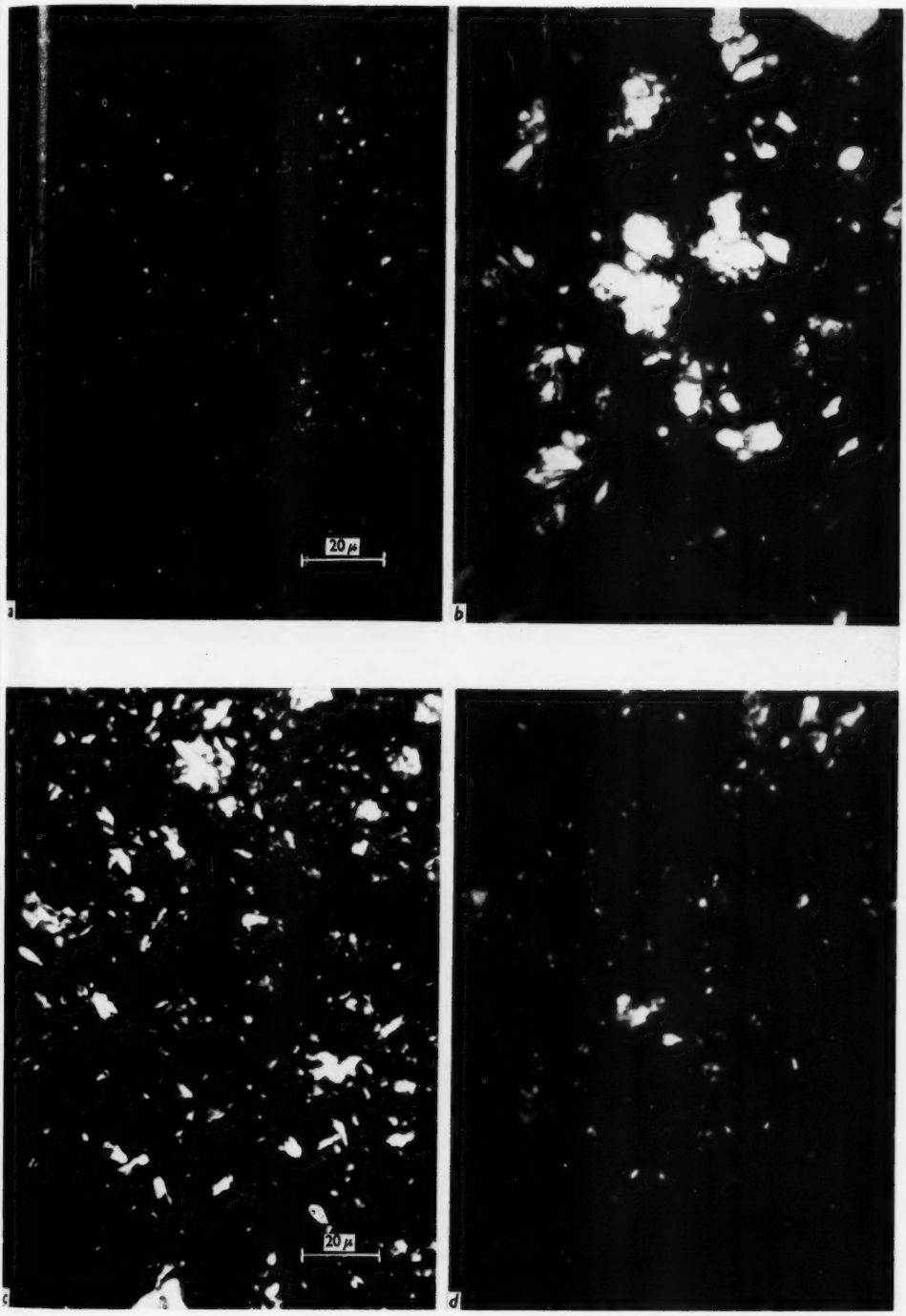
(*a*) Polarized light photomicrograph of crystals in rapidly cooled milk fat.

(*b*) Polarized light photomicrograph of crystals in slowly cooled milk fat.

(*c*) Polarized light photomicrograph of crystals in rapidly cooled interesterified milk fat.

(*d*) Polarized light photomicrograph of crystals in slowly cooled interesterified milk fat.

Printed in Great Britain



J. M. De MAN

(*Facing p.* 122)

J. Dairy Res. (1961), **28**, 123

The effect on the amount of residual milk of the interval between milkings, duration of milking, and delay between udder stimulation and milking

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(*Received* 10 *November* 1960)

SUMMARY. Using an experimental method of half-udder comparisons the effects on the amount of residual milk of varying milking intervals, the duration of milking, and the delay between the stimulation of milk ejection and the start of milking were measured. Yield of residual milk was not related to the length of milking intervals within the range of 4–16 h but the butterfat content was directly related to the length of the interval. Neither the yield nor the butterfat content of residual milk was affected by a change in the duration of milking of 1 min. A delay between the stimulation of ejection and the start of milking of 6–8 min resulted in increasing the yield of residual milk by about 1 lb.

It is suggested that these results indicate that milk ejection is sufficiently persistent that residual milk is unlikely to be affected by delays between stimulation of milking and the start of milking until they exceed 4–6 min.

It has been suggested that in cases where long milking intervals, slow milking, or a delay between stimulation and milking depress milk yields, it may be because they increase the amount of residual milk left after each milking (see the review by Elliott, 1959). The only evidence to support this has been that of Miller & Petersen (1941) and Dodd, Foot & Henriques (1949) who demonstrated an increase in residual milk when the interval between the stimulation of ejection and the start of milking is delayed, and Turner (1953) who found a connexion between the length of the milking interval and residual milk.

The three experiments described below were planned to investigate in turn the effect on amount of residual milk of length of milking interval, duration of milking, and of a delay between udder stimulation and milking. It was hoped that they would also indicate a method of controlling, for experimental purposes, the amount of residual milk.

EXPERIMENTAL

Residual milk amount was measured by remilking after injecting 10 i.u. oxytocin hormone through a mammary vein canula in the way described by Elliott, Dodd & Brumby (1960). An interval of 48 h was left between oxytocin injections to allow

* Animal Health Trust Wellcome Research Fellow, 1956–58.

residual milk to recover to its normal amount and butterfat content. Eleven Ayrshire heifers and one Shorthorn cow were used but not all for each of the three experiments. The main reason for loss of animals during an experiment was unserviceability of a mammary vein canula, if this were discovered too late for a replacement canula to be inserted before an injection was due.

Experiment 1. *The effect of the length of the milking interval on amount of residual milk*

One Shorthorn cow and nine Ayrshire heifers were placed on this experiment but complete results were obtained with only seven Ayrshire heifers. The mean 12-hour milk yields of these seven heifers was 19 lb. They had been in milk for 53 days on average at the beginning of the experiment.

Experimental methods

Cows were milked after intervals of 4, 6, 8, 10, 12, 14 and 16 h. Each interval was repeated at least three times in succession and residual milk removed only after the last milking. The intervals were so arranged that the series for each interval ended at 8 p.m., so that the residual milk was removed at the same time of day. The milking interval was the same for all cows at any one time, the order of the intervals being random. The order of intervals used over the total experimental period of 14 days was as follows. The interval immediately after which residual milk was removed is referred to as the measurement interval.

48-hour period	Intervals making up the 48-hour periods							
1	12	12	12	12	—	—	—	—
2	12	12	6	6	6	6	—	—
3	10	8	10	10	10	—	—	—
4	12	12	8	8	8	—	—	—
5	6	14	14	14	—	—	—	—
6	12	12	4	4	4	4	4	4
7	16	16	16	—	—	—	—	—

Milking

All milking and stripping was done by machine, and udder washing of each cow was done immediately before milking. At the milkings when residual milk was to be measured, as soon as stripping was completed, the machine was removed, an injection of 10 i.u. Pitocin (Parke Davis) made, and the machine replaced without delay.

The yield of milk was determined at every milking by weighing. Butterfat percentage determinations were made of the milk obtained after the interval immediately preceding the measurement interval, after the measurement interval, and of the residual milk. Milking rates were not recorded during the experiment, but were taken soon afterwards for each animal after milking intervals of 15 and 9 h duration.

Results and discussion

The mean milk yield and butterfat percentage during each of the seven preliminary and immediately subsequent measurement intervals and the residual milk yield and butterfat percentage are shown in Table 1.

The residual milk yields and butterfat percentages for the individual animals are given in Table 2, together with the significance of the treatment differences.

Table 1. *Mean milk yields and butterfat percentage for milking intervals of* 4–16 *h*

Interval, h	Preliminary int.		Measurement int.		Residual milk	
	Yield, lb	Fat, %	Yield, lb	Fat, %	Yield, lb	Fat, %
4	8·7	3·3	7·1	3·5	1·2	8·5
6	10·0	3·7	12·0	3·5	1·7	8·8
8	12·5	3·4	15·0	3·8	2·0	11·6
10	16·1	4·0	15·6	4·0	1·9	12·9
12	17·8	3·9	20·3	4·0	1·8	13·1
14	17·8	3·8	21·0	4·1	1·8	13·6
16	22·3	3·2	23·0	3·6	1·7	13·8

The average time from putting cups on until stripping point for these cows after intervals of 9 and 15 h was as follows

Interval, h	Yield, lb	Time, min
9	14·2	4·1
15	23·2	5·0

Table 2. *The yields and butterfat percentages of residual milk from individual cows for intervals of* 4–16 *h*

Cow no.		Milking interval, h						
		4	6	8	10	12	14	16
51	Yield, lb	1·2	2·1	2·2	1·5	1·6	1·7	1·1
	Fat, %	9·0	7·0	10·0	11·9	12·8	12·4	12·8
52	Yield, lb	1·2	1·1	2·6	1·9	1·7	2·1	2·0
	Fat, %	7·1	6·2	12·4	11·5	10·1	13·8	13·4
56	Yield, lb	1·5	1·9	2·0	1·6	1·5	1·9	2·7
	Fat, %	8·0	8·7	9·8	10·4	12·6	13·0	12·7
57	Yield, lb	0·8	1·9	1·8	2·4	1·7	1·5	1·2
	Fat, %	10·5	12·9	14·1	14·9	14·8	16·8	16·3
58	Yield, lb	0·8	1·0	1·3	1·5	2·2	1·8	1·3
	Fat, %	7·4	8·4	11·9	13·3	14·9	14·7	12·4
59	Yield, lb	1·8	1·9	2·0	2·2	2·3	1·8	1·5
	Fat, %	9·2	9·6	11·6	13·1	13·5	12·3	12·7
62	Yield, lb	1·3	1·9	2·1	2·3	1·9	1·9	2·3
	Fat, %	8·0	8·6	11·6	14·9	13·3	12·4	16·2
Mean	Yield, lb	1·2	1·7	2·0	1·9	1·8	1·8	1·7
Mean	Fat, %	8·5	8·8	11·6	12·9	13·1	13·6	13·8

Standard error of the difference in milk yield between any two means = 0·20 lb.
Required difference between any two means to reach significance at the 5 % level = 0·45 lb (using method of Tukey adapted by Snedecor, 1956).
(Error percentage = 22.)
Standard error of the difference in butterfat percentage between any two means = 0·61.
Required difference between any two means to reach significance at the 5 % level = 1·3 (Snedecor, 1956).
(Error percentage = 10.)

The amount of residual milk was significantly lower after the 4-hour interval than after any other interval, otherwise it was unaffected by length of interval or amount of milk in the udder at milking over the range occurring in the experiment, namely

4–16 h intervals and 7–23 lb of milk. A greater difference might have occurred had the experiment not been confined to fast-milking heifers with very low residual milk yields. The residual butterfat percentage, on the other hand, showed a rise with interval length which was very marked, and significantly so over the shorter intervals.

This experiment points to a need to investigate further Turner's (1953) claim of a positive relationship between residual milk amount and interval length.

Experiment 2. The effect of duration of milking on residual milk and butterfat yield

In this experiment the duration of milking (the time from putting on the cups to stripping) was varied by the use of different vacuum levels of 15 and 18 in. Hg in conjunction with pulsation ratios of 1:1 and 3:1, respectively.

Eleven of the thirteen animals available were used and ten completed the experiment. The cows had been in milk for an average of 77 days and the average yield when milked at 12-hour intervals was 18 lb at the beginning of the experiment.

Experimental design

The treatment—slower milking—was applied to one-half of the udder at the same time as the other half was being milked at the faster rate. Immediately after the treatment, an injection of 10 i.u. oxytocin (Sandoz Syntocinon) was given to the cow and the residual milk was removed from both udder-halves. The slower milking treatment was applied to each animal at intervals of 48 h four times in all, and to each udder-half alternately, half the cows receiving the slower milking first on the right half and the others first on the left half. The cows were milked twice a day at equal intervals. Half the cows received the slower milking treatment at a morning milking and half at an evening milking.

Milking

All milking and stripping was done by machine. A half-udder machine was used so that milking of each half could proceed independently. The residual milk was extracted from both udder halves at the faster rate. Each cow's udder was washed immediately before milking. At the treatment milking the machine was removed from each half as milking finished. It was replaced on both halves immediately after the injection of oxytocin.

The yield was determined at all milkings by weighing. Samples for butterfat testing were taken from the residual milks only.

The duration of milking was measured from each udder-half at all treatment milkings.

Results and discussion

The time from beginning of milking to beginning of stripping for the faster and slower milking was 4 and 5 min, respectively.

The mean amounts of residual milk and their butterfat percentages at the end of faster and slower milking are given in Table 3.

The slower milking, which caused a difference in milking time of 1 min, had no effect on the yield of residual milk or its butterfat content.

Table 3. *Mean yields and butterfat percentages of residual milk for half udders milked at two rates*

	Yield, lb	Fat, %
Slower milking	0·98	11·8
Faster milking	0·97	11·2
Difference (in favour of slower)	—	+0·6
Standard error and significance of the difference	—	0·8, N.S.

Experiment 3. *The effect of varying the length of time between stimulation of the udder and application of the milking machine on the yields of residual milk and butterfat*

The length of the delay between udder stimulation and milking which was investigated ranged from 0 to 10 min.

Of the twelve cows used for this experiment only one failed to complete the treatments. At the beginning of the experiment the eleven cows had been in milk for 65 days on average and their mean 12-hour milk yield was 18 lb.

Experimental design

The design used was a duplicated 6×6 incompletely balanced Latin square. The treatments were periods of delay of 0, 2, 4, 6, 8 and 10 min between stimulation and milking. These treatments were applied to one-half of the udder only, the other half (the control half) being milked immediately after stimulation of let down as was normal for these cows. The treatment half was the right half for one group of six cows, and the left half for the other group, and remained so throughout the experiment. The treatments were spaced 48 h apart, all other milkings taking place immediately after stimulation. The residual milk was removed from both halves of the udder after every treatment milking. A single injection of 10 i.u. oxytocin (Sandoz Syntocinon) was given.

Milking

All milking and stripping was done by machine. A half-udder machine was used so that the right and left halves of the udder could be milked at different times when necessary. At the treatment milking the whole udder received the normal washing stimulus and two teatcups were attached to the control half. The other half of the udder was milked after the allotted treatment delay. Each half of the udder was stripped separately and the half machine was removed as soon as stripping was completed. When milking was completed the oxytocin injection was given, and the half machines replaced immediately.

The yield of milk of each udder half was determined by weighing at each milking. Samples for butterfat testing were taken from the treatment milkings and from the residual milk only.

Results and discussion

The mean yields of residual milk and butterfat, and the percentage of butterfat in the residual milk obtained from the control and treatment udder halves of the

eleven cows after the six treatments are shown in Table 4. The missing cow in one Latin square has been replaced by its duplicate from the other.

The complete residual milk data for the eleven individual animals are shown in Table 5.

Table 4. *Mean yields of milk and butterfat, and butterfat percentage of the residual milk of half-udders*

	Delay between stimulation and milking, min					
	0	2	4	6	8	10
Milk, lb						
Delayed half	1·1	1·2	2·2	2·4	2·4	3·0
Control half	1·1	1·2	1·8	1·6	1·7	1·4
Difference	0	0	0·4	0·8	0·7	1·6
Level of significance of difference	N.S.	N.S.	N.S.	1%	1%	0·1%
Butterfat, lb						
Delayed half	0·14	0·15	0·25	0·26	0·25	0·31
Control half	0·13	0·14	0·19	0·17	0·18	0·18
Difference	0·01	0·01	0·06	0·09	0·07	0·13
Butterfat, %						
Delayed half	13	13	11	11	10	10
Control half	12	12	11	11	11	13

Table 5. *Difference between udder halves in the amount of residual milk expressed as additional residual milk (lb) in the half milked after a delay*

Cow no.	Duration of delay in milking, min					
	0	2	4	6	8	10
57	0·1	−0·1	0·3	1·3	−0·2	2·0
60	0·0	0·0	1·0	1·3	0·8	0·9
54	0·1	−0·1	0·3	−0·3	0·9	4·2
59	0·0	0·0	−0·9	0·5	0·0	1·9
58	0·0	0·3	1·2	2·3	0·6	0·9
11	0·3	−0·2	0·3	0·7	1·5	1·6
55	0·4	0·1	0·1	0·8	1·3	1·1
56	−0·2	0·0	0·3	0·0	0·2	0·6
52	0·0	0·0	0·7	0·9	2·1	2·9
62	−0·1	0·0	0·1	0·4	0·3	−0·5
51	0·0	0·1	0·5	0·4	0·5	1·8
Mean after substitution of no. 57 for missing cow	0·06	0·01	0·4	0·8	0·7	1·6

It would seem that the amount of residual milk left in the udders of these cows was significantly affected by a delay between udder stimulation and milking only when this was longer than 4 min. With delays over 4 min the effect increased the longer the delay up to the longest used which was of 10 min. It can therefore be supposed that for these cows the milk ejection response did not begin to fade appreciably until after a period of time equal to milking time plus about 4 min (cf. Dodd *et al.* 1949). The response appeared to be fairly consistent with only three or four instances of a second milk-ejection reaction during the milking of the delayed half (Table 5).

GENERAL DISCUSSION AND CONCLUSIONS

In these three different investigations into residual milk the amount was not altered by the length of the milking interval, nor by a milking procedure which took 1 min longer than usual. It was, on the other hand, increased by leaving a delay of 4–6 min between udder stimulation and the beginning of milking. It is likely that the underlying reason for all three results is the high persistence of the milk ejection reaction, and that residual milk amount would be increased only by a treatment involving an increase of more than 4 min over and above normal milking time. The fact that neither the longest milking interval used nor the slow milking increased the duration of milking by more than 1–2 min, accounts for the ineffectiveness of these treatments in causing a change in the amount of residual milk.

The fat content of residual milk was, on the other hand, affected by the length of the milking interval, but was not altered by a delay between udder stimulation and milking. A higher butterfat percentage in the residual milk after longer intervals was noted by Johansson (1952), who also suspected that there was a higher butterfat percentage in the residual milk after slower extraction of milk. The difference found after the slower extraction in Expt. 2 cannot be used to support this observation as it was not significant.

The technique of delayed milking would seem to offer a possibility of experimentally controlling the amount of residual milk left after a milking. In particular, when the milking of a half-udder is delayed with the other half always milked normally it would seem possible to control residual milk consistently without fear of the cow adapting her milk ejection reaction.

For the cows on this experiment, an increase of approximately 1 lb of residual milk in one udder half could be achieved by delaying its milking for 6 min.

I wish to thank Dr F. H. Dodd for his advice and help.

REFERENCES

Dodd, F. H., Foot, A. S. & Henriques, E. (1949). *J. Dairy Res.* **16**, 301.
Elliott, G. M. (1959). *Dairy Sci. Abstr.* **21**, 435, 481.
Elliott, G. M., Dodd, F. H. & Brumby, P. J. (1960). *J. Dairy Res.* **27**, 293.
Johansson, I. (1952). *Acta agric. scand.* **2**, 82.
Miller, K. & Petersen, W. E. (1941). *J. Dairy Sci.* **24**, 225.
Snedecor, G. W. (1956). *Statistical Methods*, p. 251. Ames: Iowa State College Press.
Turner, H. G. (1953). *Aust. J. agric. Res.* **4**, 118.

Printed in Great Britain

J. Dairy Res. (1961), **28**, 131

The estimation of blood in bovine milk

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(*Received* 14 *November* 1960)

SUMMARY. Details are given of a method, based on the peroxidase activity of the sedimented erythrocytes, that is suitable for the quantitative estimation of blood in milk.

Investigations described elsewhere (Rammell, 1961) called for a simple, routine method for the examination of many milk samples for blood content. The method described here was devised primarily for this purpose.

METHODS

Existing methods

Past literature was extensively searched for details of a method for the accurate estimation of blood in milk, but without success. Indeed, references to blood in bovine milk are comparatively rare, being usually confined to milk visibly stained with blood (Blackburn & Macadam, 1954), or to the near absence of erythrocytes in normal milk (Varrier-Jones, 1924). The overall cell content of milk has been quantitatively estimated by examination of stained, Breed-type smears of whole milk (Blackburn & Macadam, 1954) or of smears of the deposit from centrifuged milk (Zlotnik, 1947). Such methods are open to serious difficulties and inaccuracies if applied to the estimation of blood in milk. Trainer (1943) refers to the use of the peroxidase activity of blood for its detection in milk, but records no quantitative method.

Laboratory tests are readily available for the detection of occult blood in certain pathological conditions of man, but these tests, based on peroxidase activity, are rarely more than semi-quantitative. The choice of reagent for use in the peroxidase reaction has varied, the most popular being guaiac, benzidine, *o*-dianisidine, and *o*-tolidine. Fielding & Langley (1958) have described a method for the quantitative estimation of haemoglobin in body fluids, using a commercial preparation containing *o*-tolidine.

Requirements of a suitable method

Centrifuging of suppliers' milk samples indicated the necessity for a method able to estimate concentrations of blood ranging from about 75 ppm. down to 2 ppm. and less. Daily examination of many milk samples was envisaged, so the tests had to be completed in under 24 h. Any peroxidase reaction that might be used must

distinguish between lactoperoxidase and haemoglobin, and preferably should be carried out in a clear medium suitable for colorimetric estimation. These requirements were met by utilizing the peroxidase reaction of the haemolysed, sedimented erythrocytes.

Final proposed method

Centrifuge tubes. Tubes (supplied by Charles Hearson and Co., London) of 25 ml capacity, made to the following specification are used: overall length 3·8 in.; diameter at top 0·95 in.; sides straight for 2 in., then tapered to a hemispherical base of 0·3 in. diameter. Although other types of tube may be used, experience has shown that these are the most suitable. Tubes with sharper taper give a sediment that is too tightly packed, while tubes with a flatter taper do not pack the sediment tight enough to allow the supernatant to drain off so well.

Haemolysing and coagulating solution (H—C). The sedimented erythrocytes can be haemolysed by the addition of distilled water, but such a haemolysate is clouded by the presence of residual milk protein which is not readily removable by filtration or centrifugation. Optimum coagulation and corresponding sedimentation of this protein can be achieved by incorporating 0·2 % (w/v) calcium chloride and 0·05 % (v/v) rennet (N.Z. Rennet Co.) in the distilled water used for haemolysis. A stock solution of this concentration is kept at 0–1 °C.

o-Tolidine reagent. Grind 5 g *o*-tolidine (The British Drug Houses Ltd.) in a glass mortar with 5 ml 20 % (v/v) hydrochloric acid for 15 min. Transfer the resulting paste to a 1000 ml volumetric flask, and make up to 1000 ml with distilled water, shaking thoroughly for 10 min. Add 0·5 g of animal charcoal, shake and leave overnight (18 h) before filtering into an amber bottle, for storage at 0–1 °C. The reagent is sensitive to light and should therefore be prepared in the minimum of light at all stages. A slight blue colour may be evident in the reagent after filtering, but on standing this soon disappears to give a colourless reagent.

Hydrogen peroxide solution. Hydrogen peroxide (30 %) is diluted with distilled water to a 3 % solution, using permanganate for standardization. This solution is stored in a paraffin wax container at 0–1 °C.

Routine procedure. Milk samples are centrifuged in 20 ml amounts at a relative centrifugal force of 1150 (in our laboratory 2700 rev/min) for 25 min. The fat layer which forms is gently loosened from the side of the centrifuge tube, and poured off with the fluid supernatant. The tubes are drained in an inverted position for 15 min.

While the tubes are draining, they are arranged in descending order of the apparent amount of blood in the sediment, those not showing any blood being arranged in descending order of the amount of coloured (usually brownish) sediment. After some experience, it is possible to distinguish blood sediments that require further dilution of the haemolysate in order to obtain a suitable reading on the colorimeter. Records are kept of the ranking by blood and sediment contents, and also of the amount of any dilution required by the sediments. These records are compared with the final quantitative results as a check on the consistency of the method.

One ml of H—C is pipetted into each drained centrifuge tube, using as much force as possible to break up the sediment. The sediment can usually be dispersed fairly easily by 'flicking' the tube vigorously. Any obstinate sediments are dispersed with

a fine glass rod. Tubes are stored at 0–1 °C overnight for 16 h to allow complete haemolysis of any erythrocytes present.

The following morning, an appropriate amount of H—C is added to any tubes containing haemolysates that require diluting. After vigorous 'flicking' of all the tubes, the haemolysates are transferred to 3 in. × $\frac{3}{8}$ in. tubes, and centrifuged at 2700 rev/min for 30 min: 0·5 ml quantities of the clear supernatants are then pipetted into matched 5 ml EEL colorimeter tubes in which the peroxidase reaction is carried out. Two operators are used for the following part of the procedure.

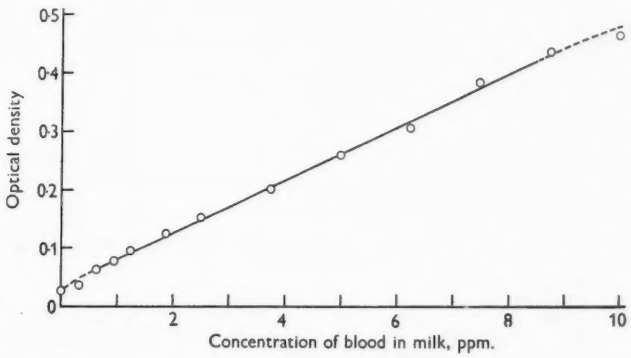

Fig. 1. Relationship between blood concentration in milk and optical density after reaction of 0·5 ml of haemolysate with 3 ml *o*-tolidine reagent and 0·5 ml of 3 % hydrogen peroxide for 6 min at 19–20 °C.

One operator adds 3 ml of *o*-tolidine reagent followed by 0·5 ml of 3 % hydrogen peroxide solution. The mixture is immediately inverted twice, the colour developed for 6 min, and the optical density then determined by a second operator using an EEL colorimeter with red (OR 1) filter, or other suitable instrument, and a distilled water blank. The first operator continues to add reagents to further haemolysates at 3 min intervals. With experience, and the use of three instead of two stop-clocks, these intervals may be reduced to 2 min, giving a through-put of thirty samples per hour. Further reduction of the time interval is possible, though hazardous. The colorimeter reading is converted to ppm. of blood by reference to the standard curve.

Preparation of a standard curve. Standard dilutions (0–10 ppm.) of citrated cow's blood are prepared in milk free from indigenous blood, or in milk from which the blood cells have been removed by centrifuging. Considerable care must be taken in the preparation of these dilutions because of the nature of the diluting fluid, milk. Twenty-ml samples of the dilutions are centrifuged and treated as described earlier under routine procedure. The colorimeter readings thus obtained are plotted against ppm. blood in the milk (Fig. 1). There is an approximate linear relationship between colour development and blood content of the milk for blood concentrations of 0·5–8·5 ppm. As the method measures primarily haemoglobin concentration, the standard curve refers to whole blood similar in its haemoglobin content to that of the blood used for the standard. The haemoglobin content of this 'standard blood' may be determined by other established methods (Wintrobe, 1951).

RESULTS

Stability of o-tolidine. For maximum stability, the *o*-tolidine should be stored at low temperatures (0–1 °C) in the dark. This also applies to the *o*-tolidine reagent which, under these conditions, remains stable for at least several months. For accurate work, a new standard curve should be prepared for each new batch of reagent.

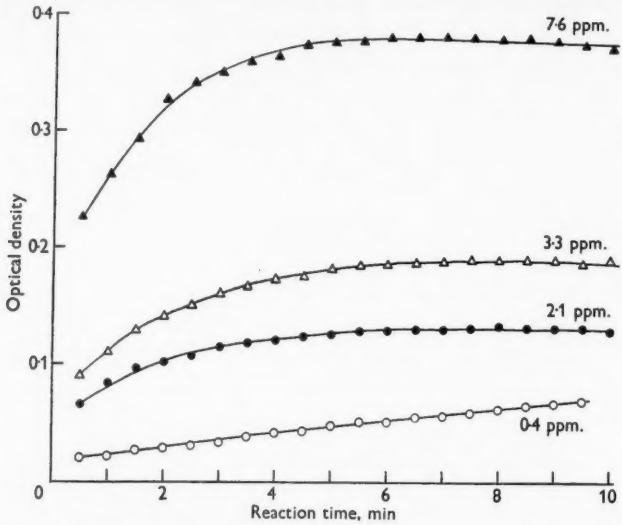

Fig. 2. Effect of reaction time on optical density of colour produced by mixing 3 ml of *o*-tolidine reagent and 0·5 ml of 3% hydrogen peroxide with haemolysates (0·5 ml) containing four concentrations of blood.

Reaction time. The effect of reaction time on colour development is shown in Fig. 2 which clearly demonstrates the need for a standard reaction time in the routine procedure. Greater accuracy would presumably be obtained with a reaction time of 12 min.

Reaction temperature. In the absence of haemoglobin, and in the range of 16·8–33·8 °C, the logarithm of the density of colour varied inversely as the absolute reaction temperature (Fig. 3). This relationship probably applied at lower temperatures also, but the colorimeter used was not accurate enough for the slower reaction rates. The inverse relationship enables the temperature coefficient (Q_{10}) of the reaction to be calculated, if we assume that the colour developed after 6 min is a direct measure of the reaction rate, and the Beer–Lambert laws apply—a reasonable assumption at such low concentrations of oxidized *o*-tolidine. The value of Q_{10} so obtained was 2·54 and this indicates that the slight oxidation occurring in the absence of added haemoglobin is purely chemical, the *o*-tolidine presumably being directly oxidized by the hydrogen peroxide. The use of a reagent sensitive to traces of catalyst thus results in an unavoidable slight oxidation of the *o*-tolidine on the addition of hydrogen peroxide and distilled water.

In the presence of haemoglobin (Fig. 3), the effect of reaction temperature is considerably reduced, the calculated Q_{10} being 1·22 and 1·18 for blood concentrations

of 50 and 100 ppm., respectively—equivalent to 2·5 and 5·0 ppm. blood in the original milk. Such a reduction in the temperature coefficient is typical of an enzyme catalysed reaction (Bull, 1945). Temperatures in excess of 25 °C give erratic results in the presence of blood, presumably due to breakdown of the haemoglobin by hydrogen peroxide. It is to be expected that the temperature coefficient will increase with decreasing concentrations of blood owing to the increasing importance of the purely chemical reaction with a Q_{10} of 2·54. In practice, this means that stricter temperature control is necessary at low concentrations of blood, and that a low reaction temperature is preferable.

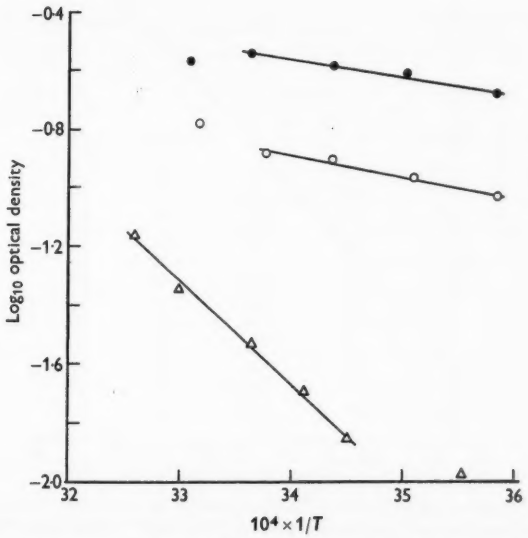

Fig 3. Effect of reaction temperature on optical density of colour produced by mixing 3 ml *o*-tolidine reagent and 0·5 ml hydrogen peroxide with 0·5 ml of distilled water (△—△), of 50 ppm. bovine blood in water (○—○), and of 100 ppm. bovine blood in water (●—●). Reaction time of 6 min. Log optical density is plotted against reciprocal of absolute temperature to yield a straight line relationship.

Interference due to lactoperoxidase. Lactoperoxidase catalyses the oxidation of *o*-tolidine by hydrogen peroxide, and so it was necessary to estimate the influence any traces of lactoperoxidase remaining in the final haemolysate might have on the results. Most of the lactoperoxidase is discarded with the supernatant milk, but a small amount remains with the sediment even after draining the tubes. The extent to which residual supernatant can influence the results is shown in Fig. 4. The interference will also vary according to the lactoperoxidase content of the whole milk.

Correlation between blood seen and blood found. The examination of sediments for visible blood after centrifuging the milk was a useful practical check on the method. This is illustrated in Fig. 5, based on the examination of 912 milk samples from sixteen suppliers to one cheese factory. Fuller details of these and other milks will be given in a later paper. With experience, it was possible to detect quite low concentrations of blood (about 2 ppm.) in 20-ml samples of milk, although false positives were

sometimes obtained visually by new personnel owing to the presence of extraneous matter having a similar appearance to blood.

Reproducibility. Attempts to measure the error of the method demonstrated the importance of ensuring homogeneity of any blood in the milk before sampling (Table 1). Visual examination of the sediments before haemolysis showed that the

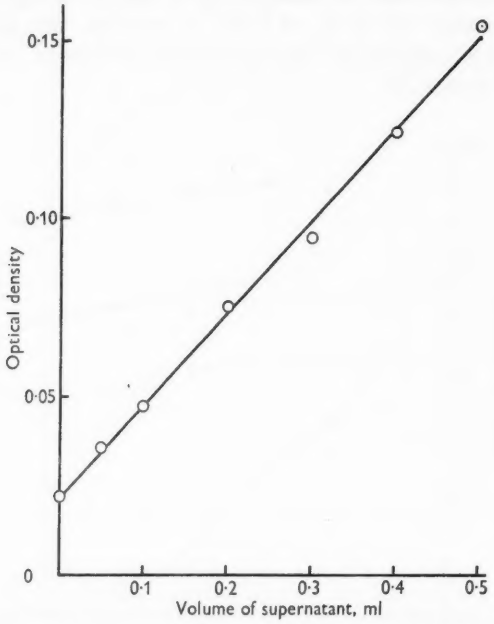

Fig. 4. Effect of residual supernatant milk on oxidation of *o*-tolidine by hydrogen peroxide. Given volume of supernatant was added to 1 ml of distilled water, and 0·5 ml of resultant mixture added to 3 ml of *o*-tolidine reagent and 0·5 ml of 3 % hydrogen peroxide. Reaction time of 6 min.

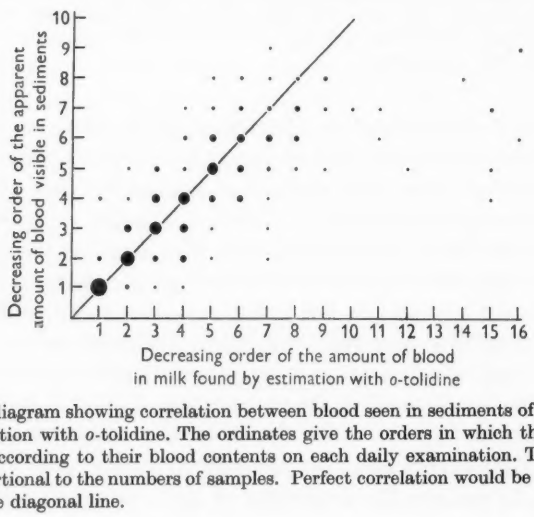

Fig. 5. Scatter diagram showing correlation between blood seen in sediments of milk and blood found by estimation with *o*-tolidine. The ordinates give the orders in which the sixteen milks were arranged according to their blood contents on each daily examination. The areas of the circles are proportional to the numbers of samples. Perfect correlation would be shown if all the circles fell on the diagonal line.

Table 1. *Blood determinations on twelve replicate 20-ml samples taken from 450 gallons of milk containing added blood, before and after flash pasteurization at 150 °F*

Sample no.	Before pasteurization, ppm. blood	After pasteurization, ppm. blood
1–4	39, 36, 30, 32	42, 43, 41, 42
5–8	32, 35, 23, 32	39, 43, 42, 44
9–12	24, 23, 19, 22	42, 43, 40, *
Average	29	42

* Tube broken in centrifuge.

Table 2. *Blood determinations, expressed as ppm., on thirty-six duplicate 20-ml samples of milk taken from 800-gallon vats of milk*

	Sample			Sample			Sample	
Vat no.	A	B	Vat no.	A	B	Vat no.	A	B
1	0·9	1·1	13	8·6	8·7	25	2·3	2·0
2	3·9	4·2	14	4·1	4·4	26	6·1	5·2
3	2·1	2·1	15	1·7	1·7	27	6·3	6·4
4	7·0	6·0	16	0·3	0·6	28	10·2	10·6
5	1·2	1·4	17	5·3	5·4	29	0·9	1·0
6	4·0	3·2	18	3·3	3·3	30	8·5	9·1
7	1·1	0·9	19	1·0	1·0	31	15·4	13·3
8	6·9	6·9	20	6·0	6·1	32	0·5	0·6
9	3·4	3·2	21	1·2	1·4	33	2·8	1·8
10	1·0	1·7	22	5·0	4·9	34	3·4	3·6
11	1·1	1·2	23	1·7	2·1	35	2·7	3·4
12	1·1	0·9	24	3·6	4·3	36	4·9	6·9

variation in the results obtained for the blood content of the milk before pasteurization was actually due to heterogeneity of the added blood in the milk. In contrast, the blood cells became fairly evenly distributed throughout the milk during pasteurization, without being lysed and without the haemoglobin activity being adversely affected. The apparent increase in activity on pasteurization is being investigated further.

Table 2 presents the results of a series of replicate determinations on thirty-six vats of cheese milk. Considering the small size of each sample (1/200 000 of the bulked milk), the agreement between duplicate samples is quite good.

DISCUSSION

Although the final method described was designed for the daily examination of many milk samples, it is readily adaptable to more refined uses of greater accuracy. The slight inaccuracies of the method, although of minor importance in obtaining an overall picture of the blood content of milk supplies, can be considerably reduced by the following modifications; these, however, may also often increase the time required for individual determinations: (*a*) double the reaction time, and so work on the 'flat portions' of the curves shown in Fig. 2; (*b*) increase the sample size when the blood concentration is low, and so obtain greater concentration of the erythrocytes; (*c*) after discarding the fat and supernatant from the centrifuged milk, wash the deposit rapidly with a slightly hypertonic salt solution to remove any residual soluble lactoperoxidase, taking care that no haemolysis of cells occurs—the widely

held view that erythrocytes are not haemolysed by hypertonic salt solutions is incorrect; (*d*) use a spectrophotometer at optimum absorption.

Although the attainment of greater accuracy is usually desirable, there may be a limit above which increased accuracy has little practical significance, because of the variation in the haemoglobin content of the blood from cows of different breeds and even from the same cow at different stages of its life. Thus, the blood content of milk expressed as ppm. whole blood is arbitrary, being determined solely by reference to the peroxidase activity of the haemoglobin released from the erythrocytes. The assumption that the peroxidase activity of haemoglobin from different cows is the same is reasonable, but has not yet been established. The expression of the blood content of milk in terms of equivalent peroxidase activity of the lysed erythrocytes would be more correct, but has little else to recommend it for practical purposes.

The difficulties encountered in routine determinations of the erythrocyte and haemoglobin content of human blood have been well studied. Wintrobe (1951) states that statistical analyses indicate that even in the hands of trained technicians the probable minimum error of red cell counts on whole blood is 7·8%, the error frequently being much greater than this. Obviously then, any method for the estimation of blood in milk based on the counting of red cells present will have an error considerably in excess of 7·8%, owing to the nature of the suspending fluid, presence of blood clots, possible agglutination of cells, and their low concentration. By indirectly measuring the haemoglobin content of the sedimented, intact erythrocytes in the milk, the errors due to uneven distribution of cells in the sample are eliminated. The method does not, however, measure any erythrocytes that may have been haemolysed before centrifuging the milk. Errors due to uneven distribution of cells in the bulk of milk can only be overcome by taking replicate samples.

The lack of references to the estimation or even presence of blood in milk has presumably been due either to the failure to recognize the extent to which cow's milk may contain blood, or to the belief that the blood content of milk has little or no significance. Unless care is taken to ensure the absence of blood in milk, some doubts may be cast upon conclusions based upon the varying peroxidase (Aurand, Roberts & Cardwell, 1956) or pseudo-peroxidase (National Institute for Research in Dairying, 1959) activities of milk. Data that are being obtained in this laboratory on the occurrence and significance of blood in milk will be discussed in a later paper.

The technical assistance of Miss Joan Vickers and Mrs A. Splite in this work is gratefully acknowledged. Line drawings were prepared by Mr A. Bryant.

REFERENCES

AURAND, L. W., ROBERTS, W. M. & CARDWELL, J. T. (1956). *J. Dairy Sci.* **39**, 568.
BLACKBURN, P. S. & MACADAM, I. (1954). *J. Dairy Res.* **21**, 31.
BULL, H. B. (1945). *Physical Biochemistry*, p. 55. New York: John Wiley and Sons, Inc.
FIELDING, H. E. & LANGLEY, P. E. (1958). *Amer. J. clin. Path.* **30**, 528.
NATIONAL INSTITUTE FOR RESEARCH IN DAIRYING (1959). *Annual Report*, p. 63.
RAMMELL, C. G. (1961). *J. Dairy Res.* **28**, 33.
TRAINER, M. T. (1943). *Proc. Soc. exp. Biol., N.Y.*, **52**, 104.
VARRIER-JONES, P. C. (1924). *Lancet*, no. 207, p. 537.
WINTROBE, M. M. (1951). *Clinical Hematology*. London: Henry Kimpton.
ZLOTNIK, I. (1947). *J. comp. Path.* **57**, 196.

Printed in Great Britain

J. Dairy Res. (1961), **28**, 139
With 1 *plate*

Fat oxidation in Cheddar cheese

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(*Received* 5 *December* 1960)

Summary. Fat and carotene oxidation in Cheddar cheese may give rise to bleached areas surrounding slits in the cheese and to a tallowy flavour. It was shown that direct contact of the fat with atmospheric oxygen was essential for oxidation to occur, and examination of cheeses showed that slits running from the rind into the interior provided this contact. Factors enhancing slit formation such as gas production by bacteria and mechanical shock to the cheese were shown to increase the incidence of tallowy discoloration. Factors which tended to exclude oxygen such as waxing the cheese surface, wrapping the cheese in plastic film or sealing in a tin were shown to reduce the incidence of the fault or eliminate it.

Accelerating factors were shown to be low cheese storage temperature, excess moisture in the cheese and possibly low salt content. Various factors which may be relevant to a commercial cure for discoloration are also discussed.

Since butterfat contains a significant proportion of glycerides in which unsaturated fatty acids occur, the fat is inherently liable to oxidation in the presence of air with the formation of fat peroxides and, subsequently, aldehydes, ketones and other degradation products. These reactions result in a typical tallowy odour and flavour. Objectionable tallowy flavours are well known as commercial faults in liquid milk, in butter, and in whole milk powder but they are not so often encountered in Cheddar cheese and there are no references in text-books to a fault in cheese which manifests itself by a change in colour and a tallowy flavour in parts of the cheese. Fat oxidation may occur in Cheddar cheese which has been stored for a long period, usually more than 8 months. Consequently when a cheese market is over supplied and some cheese is held for long periods the oxidation fault has at times given rise to widespread complaints by merchants and consumers. On the basis of commercial experience cheeses made from raw or flash pasteurized milk seem to be equally susceptible.

Tallowy discoloration in Cheddar cheese shows itself as bleached areas in the vicinity of slits in the cheese body and the discoloured portions have a tallowy flavour. The bleaching is due to oxidation of the carotene which accompanies oxidation of the fat. The fault has sometimes been called 'white streak' but since bleaching can occur through excessive acidity in cheese without the tallowy flavour, the

* Professor Riddet died on 30 December 1958.

description 'tallowy discoloration' is more specific. Moreover, the more serious aspect of the fault from the consumer's point of view lies in the tallowy flavour rather than in the bleached colour. Pl. 1*a*, *b* shows the appearance of the cut surfaces of a normal cheese and of a cheese from the same vat in which discoloration has developed.

Another form of discoloration, sometimes called 'muddy discoloration', appears to be a forerunner of tallowy discoloration although this has never been finally proved. The 'muddy' areas are not, however, objectionable in flavour. For the sake of clarity, 'muddy discoloration' is not considered in this paper.

Investigations of tallowy discoloration have been made at this Institute over many years. The fact that the fault usually takes 9–12 months to become manifest means that experimental work is very slow. The following account gives the evidence which has been adduced on the nature of the defect and mention is made of possible methods of prevention. Part I deals with the role of oxygen and part II with accelerating and retarding factors.

PART I. ACCESS OF OXYGEN IN RELATION TO DISCOLORATION

Although the tallowy flavour in discoloured cheese is proof in itself of a fat oxidation process, only after some investigation was it realized that access of atmospheric oxygen is necessary for the development of discoloration in ripening cheese. Much of the evidence which is described in the following sections supports the hypothesis that any factor which increases access of atmospheric oxygen to moist cheese surfaces promotes the development of tallowy discoloration. If this hypothesis is correct it follows that one method of preventing the occurrence of discoloration lies in exclusion of oxygen.

Association of discoloration with slits in the cheese body

Cheddar cheese is ideally a cheese with a completely 'close' texture. The ideal is, however, rarely achieved, and there are almost always 'mechanical' holes or 'slits' in the cheese body. Mechanical holes are either the result of failure to press the curd particles together or are the result of gas formation very early in the life of the cheese (perhaps even in the press), but slits are formed somewhat later as a result of the formation of gas by bacteria after the curd particles have fused together. Cheeses which are quite close in texture when 2–3 weeks old often develop slits during a 6-month curing period. The bleached zones in a discoloured cheese always occur around slits—the so called 'white streak' has a slit as its centre line. But it is not always easy to observe that such a slit on a cut surface of a cheese has a connexion with the atmosphere. Careful dissection of an 80-lb cylindrical cheese is necessary to demonstrate that this is so since the slit observed on the cut surface may connect with the rind in a different plane. Moreover, the slit as it reaches the rind of the cheese may form a crack so fine that it is difficult to detect by eye. Evidence obtained by dissection indicated that slits with discoloured areas around them always extended to the rind of the cheese, but the association of atmospheric oxygen with discoloration obviously needed more stringent proof.

Influence of bacteria

In early experiments in the 1930's it was found that addition to cheese milk of an emulsion of discoloured cheese or of a mixed culture of bacteria from discoloured cheese caused an earlier development of discoloration in the experimental than in the control cheeses. Addition of a sterilized cheese emulsion did not promote discoloration. This was taken as evidence that a bacterial enzyme played a part in the fat oxidation. But more recently it was observed that all the experimental cheeses containing added living bacteria from discoloured cheese were more open in texture than the controls, and it was realized that the bacterial effect might be indirect through gas production and the consequent formation of slits through which air could gain access to the cheese surfaces along the slits. Pl. 1 c–f shows some typical control and experimental cheeses. The hypothesis that bacterial enzymes are solely and directly responsible for fat oxidation persisted for a long time and the addition of cheese emulsions to cheese milk was used as a convenient means for artificially inducing discoloration. There was an unexplained anomaly in the results of the experiments at that time in that emulsions from normal cheese added to cheese milk often had the effect of stimulating the occurrence of discoloration similar to that produced by emulsions of discoloured cheese. This fact was later seen to fit in with the hypothesis of an indirect bacterial effect through gas production and slit formation.

Effect of exclusion of air from the interior of the cheese

There are three methods of packing cheese which would (incidentally if not by design) exclude atmospheric oxygen either partly or completely from the interior of a cheese.

(i) Coating the cheese surfaces with wax. This is done primarily to reduce moisture loss and to prevent mould growth. It is a practice which has become widespread in all cheese-making countries in recent years. Mixtures of various types of wax have been devised to make the coatings more plastic and thus less likely to crack than plain paraffin wax.

(ii) Wrapping the cheese in any one of the several newly developed synthetic films. The cheese is usually made without a bandage and the wrapping is applied as the cheese comes from the press.

(iii) Sealing in a tin, a method sometimes used on a small scale for preventing moisture loss and hastening the ripening process.

Many experiments were carried out to find whether the exclusion of air by these methods eliminated or reduced the incidence of tallowy discoloration in cheese. The results all supported the hypothesis that the phenomenon is due to contact of oxygen with the cheese fat and that exclusion of air eliminates the fault in cheese.

Waxing was shown to reduce the incidence of the fault but not to eliminate it, which is understandable since even the most plastic wax is liable to lift and crack under the influence of internal gas pressure or as a result of mechanical damage. Since waxing is a practical and cheap method of treating cheese, having beneficial effects in mould control and moisture retention as well as reducing the incidence of discoloration, it was desirable to measure more accurately the extent of its effect in controlling discoloration. At this stage, therefore, arrangements were made to carry

out trials on a commercial scale, and since several of these trials will be referred to later in connexion with the effect of other factors on discoloration the general scheme adopted is described below.

Experimental method

Cheese made in a commercial factory over the course of 2–3 weeks was divided into the requisite number of strictly comparable experimental batches. Over a period of several years cheeses from factories in different districts were used for the experiments. When different manufacturing treatments were to be applied, milk was run into two vats simultaneously, cheese was made in the two vats by the methods specified, and two to four cheeses were taken at random from each vat daily for 2–3 weeks to make up the control and experimental batches. Since it had been found that discoloration did not necessarily occur in all the cheeses from any one vat because of the chance incidence of slits, the object was to use as many cheeses as practicable in each batch so as to get a statistically significant result. With the facilities available 100 cheeses per batch was about the practicable limit, so this was the aim although in the earlier experiments only fifty cheeses were sometimes used.

After manufacture the cheeses were stored in the factory curing room for 14 days, usually under controlled conditions of temperature (55 °F) and humidity (80–85 %). They were turned daily. They were then waxed (except where the experiment called for unwaxed cheese), packed in crates and sent by rail to cheese stores in Wellington or Auckland, where they were held at 45 °F (no humidity control) for 9–12 months. Towards the end of the storage period a few control cheeses were cut in half for examination (extra controls had been provided for this purpose) and when there was some assurance that a reasonable proportion (say at least 10–20 %) of the control cheeses would show discoloration, all the cheeses were removed from the crates and cut in half for examination. Usually the surfaces were photographed, a dark blue filter serving to emphasize the contrast between the normal-coloured and bleached areas. Other features of the cheese such as texture, flavour, and firmness of body were observed and recorded.

(i) *Effect of waxing*

Using the procedure described above several experiments were made to determine the effect of waxing on the incidence of tallowy discoloration. The details of two typical experiments are given below.

Discoloration in waxed and unwaxed cheeses

	Expt. 1	Expt. 2
Factory	Bruntwood	Waihi
Temperature of storage	45 °F	45 °F
Time of storage	12 months	11 months
Proportion of unwaxed cheeses discoloured	60/150	37/108
Proportion of waxed cheeses discoloured	19/150	17/108

The results indicated that waxing of the cheese surface significantly reduced the incidence of discoloration. This finding was confirmed by the results of several similar experiments.

(ii) *Effect of wrapping in plastic film*

In a commercial factory curd from some of the vats was packed as normal 80-lb bandaged cylindrical cheeses, while from other vats it was packed as 60-lb rectangular cheeses wrapped in plastic film to form the so-called rindless cheeses. In this particular experiment it was not possible to bulk all the milk before the vats were filled. Consequently the cheeses were made from milk which may have varied slightly in composition from vat to vat. However, in a second experiment at another factory arrangements were made to fill two vats simultaneously so that the cheeses were more strictly comparable. The results for both experiments were similar. Although only four of the rindless cheeses showed any internal discoloration it was observed

Discoloration in bandaged and rindless cheeses

	Expt. 1	Expt. 2
Factory	Kaupokonui	Waihakeke
Temperature of storage	45°F	45°F
Time of storage	10–11 months	12–13 months
Proportion of bandaged cheeses discoloured	35/100	27/100
Proportion of rindless cheeses discoloured	0/100	4/100

that on nearly all of them there were patches of whitish tallowy-tasting cheese on the surface immediately under the wrapper. This was in the form of a very thin layer which could be scraped off easily. The phenomenon was regularly observed with rindless cheeses stored for about 12 months in one particular film and later work indicated that its occurrence was due to the permeability of the film to atmospheric oxygen. On long storage the small amount of oxygen which passed through the film to the moist cheese surface apparently allowed oxidation of fat to occur. With films of lower permeability than that used in the early experiments the surface tallowiness did not occur.

Surface oxidation is not readily apparent on normal bandaged cheese in spite of the fact that it is completely exposed to air where the cheese is not waxed or imperfectly protected from air by a 'wax' coating. But there is also the difference that evaporation of moisture, prevented in the case of rindless cheese, causes the formation of a hard rind on bandaged cheese. Pont & Rogers (1961) found that the surface of butter oxidized less readily when it was desiccated, and the drying of the cheese surface probably accounts for the absence of obvious tallowiness on bandaged cheeses. When the bandage was stripped from some of the cheeses and the hard rind removed with a spokeshave, patches of a thin layer of bleached tallowy cheese were often apparent even though the interior of the cheeses was free from discoloration. This may have been the result of access of oxygen to the moist cheese surface before it dried out in the first weeks of the maturing process.

(iii) *Effect of sealing in a tin*

No large-scale experiments in commercial factories were carried out to compare normal cheeses with similar cheeses sealed in tins. In many small-scale comparisons made at various times over several years discoloration was never observed in mature cheeses sealed in tins within 3 weeks of manufacture. An experiment carried out at the Institute in 1955–56 gave clear-cut results. Cheese was made daily for 5 days from approximately 600 gal of the regular milk supplies. The milk was flash pas-

teurized at 150 °F and the cheese curd was packed as 40-lb cheeses. The same starters were used throughout the experiment. Cheeses made on the Monday and Tuesday were used as controls. On the following 3 days an addition of 300 g of discoloured tallowy cheese emulsified in Ringer solution was added to the cheese milk. On the first day of addition the emulsion had previously been autoclaved at 15 lb/in² for 20 min while on the last 2 days unsterilized emulsion was used. There were finally (*a*) twenty-two control cheeses, (*b*) fourteen cheeses with sterilized emulsion, (*c*) twenty-five cheeses with unsterilized emulsion. After all the cheeses had been stored at 55 °F for 14 days eight from group (*a*) five from group (*b*) and nine from group (*c*) were sealed in tins into which the cheeses fitted fairly closely, the small remaining space being filled with sawdust in the usual way. All the cheeses tinned and not tinned (and unwaxed) were stored at 45 °F for10 months and then cut for examination. The incidence of tallowy discoloration was as follows:

Effect of addition of discoloured cheese to cheese milk

	Controls		Sterilized emulsion		Unsterilized emulsion	
	Not tinned	Tinned	Not tinned	Tinned	Not tinned	Tinned
Proportion of cheeses discoloured	0/14	0/8	0/9	0/5	16/16	0/9

The cheeses in which unsterilized emulsion was present all showed more slit openness than the cheeses of the other groups, and all the tinned cheeses were fuller in flavour than the untinned cheeses. The effect of enclosure in a sealed tin in negativing the discoloration-promoting action of the unsterilized cheese emulsion was definite even though the number of experimental cheeses was small.

The influence of transport conditions

Tallowy discoloration does not necessarily occur in all the cheeses made from one vat of curd and its incidence varies between batches of similar cheeses when the batches are subjected to different transport and handling treatments. These facts constitute evidence that discoloration occurs partly as a result of variation in some physical characteristic of cheese. The following experiment was designed to clarify the effect of transport conditions.

Cheeses made at the Waihakeke factory over the course of about a month were divided into two strictly comparable batches. After storage at the factory for 14 days they were waxed and crated in the usual way. Both batches were railed to Auckland and stored at 45 °F. After 3 months one batch was shipped to England and almost immediately reshipped back to Auckland. Temperature in the ships' holds was 45 °F. Both batches were then held in Auckland at 45 °F until the cheeses were 12–13 months old, when they were cut in half and the cut surfaces examined for discoloration. Tallowy discoloration was evident in twenty-seven of the 100 cheeses stored throughout in New Zealand and in sixty-five of the 100 cheeses which had been subjected to a double transport treatment. The wax on the transported cheeses appeared 'shattered' with a multiplicity of fine cracks showing on its surface. The admission of air into the interior of these cheeses was also shown by the occasional occurrence of mould penetration along cracks into the interior of the cheese.

The results gave a clear indication that some conditions existing during transport had increased the tendency to fat oxidation, the most probable explanation being that mechanical shock had promoted the admission of air into the cheese as a result of the formation of cracks in the cheese and the shattering of the wax coating. The evidence that admission of air is the factor involved is not, however, conclusive since transport often involves a change in environmental factors as well as mechanical shock.

Considering all the experimental results so far described, there is strong evidence supporting the hypothesis that tallowy discoloration occurs as a result of the access of oxygen to the fat in a moist cheese surface such as exists in a slit in the solid body of a cheese or under a plastic film which prevents moisture evaporation from a cheese surface.

PART II. ACCELERATING AND RETARDING FACTORS IN FAT OXIDATION

Although cheese fat apparently cannot suffer oxidation in the absence of atmospheric oxygen, in the presence of oxygen it does not necessarily oxidize at a constant rate in all cheeses. Other factors may influence the rate of oxidation. The development of tallowy discoloration is a slow process and it normally takes several months for the typical bleached colour and tallowy flavour to appear. During that time conditions in the body of the cheese are changing; there is a gradual degradation of protein and the oxidation-reduction potential falls as a result of bacterial activity. The oxidation of fat in other media such as milk and butter is well known to be influenced by bacterial action, by storage temperature and by moisture and salt contents. These factors are likely to come into play in cheese as well as other factors peculiar to cheese.

Observations on some of the factors considered likely to influence fat oxidation in cheese are recorded below.

Influence of light

It had been suggested that exposure of cheese curd to sunlight during the manufacturing process might render the fat in the cheese more liable to oxidation as the cheese matured. This hypothesis was tested on three occasions by spreading curd after milling in a thin layer on a tray and exposing it to direct bright sunlight for 45 min. The curd was then salted and packed as an 80-lb cheese. The three cheeses made in this way, together with appropriate control cheeses made from similar curd not exposed to direct sunlight, were stored at 45 °F for 10 months. None of the cheeses showed any discoloration after storage but the experimental cheeses had a slight off-flavour. Thus although exposure to sunlight had apparently had some effect, which was manifested in a flavour difference, it did not appear to increase susceptibility of the cheese to fat oxidation and discoloration.

Influence of temperature of storage

Storage experiments with commercial cheeses carried out on several occasions from 1954 onwards gave results which indicated that, contrary to expectation, the development of discoloration was favoured by low storage temperatures. The following experiment indicates the type of result obtained.

Cheeses from three commercial factories were divided into three comparable batches each of fifty waxed and fifty unwaxed cheeses and stored at 33, 42 and 58 °F, respectively. After storage for 11 months the cheeses were cut and examined with the following results.

Effect of temperature of storage on incidence of discoloration

	33 °F		42 °F		58 °F	
	Waxed	Unwaxed	Waxed	Unwaxed	Waxed	Unwaxed
Proportion of cheeses discoloured	28/50	46/50	21/50	39/50	0/50	3/50

Apart from the differences in incidence of discoloration there were distinct differences in the flavours of cheeses stored at the different temperatures. Those at 33 °F had in general 'flat', uninteresting and sometimes bitter flavours; those at 42 °F tended to have mild 'cheesey' flavours without bitterness; those at 58 °F all had a fuller flavour with a 'sulphide' characteristic to which some consumers might object.

The rather unexpected temperature influence on discoloration may be explained by its influence on the oxidation-reduction potential in the cheese. Although fat oxidation might be expected to proceed more quickly at higher temperature and might possibly be stimulated by bacterial enzymes, the lowered oxidation-reduction potential produced by bacterial growth may be the overriding factor. Where cheese is stored at a low temperature bacterial action is checked and the oxidation-reduction potential tends to drift to higher levels at which fat oxidation can occur more easily. In addition to this, oxygen is more soluble at lower temperatures. Thus although in some systems (e.g. in butter) oxidation of milk fat progresses more quickly at higher temperatures, under the conditions existing in cheese the reverse is the case. It is well established that a similar phenomenon occurs in liquid milk where 'oily' flavours due to fat oxidation occur more readily when the bacterial content is low and the oxidation-reduction potential has not been appreciably lowered. It has been shown that a cheese stained on its outside surface with methylene blue and then wrapped in one of the synthetic plastic films which is relatively impervious to oxygen, tends to reduce the blue as a result of the development of a reducing potential by bacterial action (Dolby, 1959). The readiness with which a reducing potential develops varies from one cheese to another and with temperature of storage. This all fits in with the hypothesis that bacterial action exerts a retarding influence on cheese discoloration, although indirectly through carbon dioxide production resulting in slit formation, bacterial action may render cheese more liable to discoloration through admission of oxygen to the interior. Bacterial action is also variable and unpredictable in its effects from one batch of cheese to another because of the wide variation in the number and types of bacteria in ripening cheeses.

Influence of moisture and salt

As has already been mentioned, the hard rind of Cheddar cheese shows little evidence of fat oxidation although air has had free access to it. Rindless cheeses, wrapped in plastic films, have been observed to show more fat oxidation on surfaces showing free moisture. Experiments were made in an attempt to determine whether

drier cheeses of firmer body were less liable to discoloration. It is difficult deliberately to alter the moisture content of cheese without at the same time altering other factors such as acidity, salt content, and texture, which may also have a bearing on discoloration. Salt content in particular is difficult to control and thus in the trials to be described it was impossible entirely to distinguish between the possible effects of salt and moisture contents.

In one experiment three batches of reasonably comparable cheeses were made, all with a pH of about 5·0 at 14 days, and of graded moisture contents. The only other significant difference was in the batch with the highest moisture content, which also had a lower salt content. The batches of eighty cheeses were stored at 45 °F and examined after 11–12 months. The following table gives the average

Effect of moisture and salt content on discoloration

Batch	W.N.F.S.,* %	Salt in moisture, %	Proportion of cheeses discoloured
1	53·2	4·5	14/80
2	55·8	4·4	23/80
3	56·8	2·7	51/80

* W.N.F.S. = water in non-fat substance.

composition of the cheeses in each batch and the number of cheeses in which discoloration was observed. In a second experiment in which two batches of cheeses had similar moisture contents but significantly different salt contents the results were as follows.

Effect of moisture and salt content on discoloration

Batch	W.N.F.S., %	Salt in moisture, %	Proportion of cheeses discoloured
1	53·6	4·8	14/76
2	54·3	6·4	11/76

The results of the first experiment indicate that either a high moisture content or a low salt content gives a significant increase in the incidence of discoloration. The results of the second experiment where there is no significant difference in incidence of discoloration suggest that moisture content is the more important factor. This tentative conclusion needs confirmation by further experiment.

Influence of blood pigments

Rammell (1961) has shown that blood added to milk in concentrations of the order of 25–200 ppm. causes tallowy discoloration to occur in the resultant cheese much earlier and to a greater extent than in the control cheeses. The discoloration in such experimental cheeses still occurs around slits where atmospheric oxygen has been in contact with the fat. It has not yet been shown that smaller concentrations of blood of the order which may occur from time to time in normal milk supplies will significantly affect the incidence of discoloration in commercial cheese, but it is definite that haematin compounds constitute a catalytic system which will stimulate fat oxidation in cheese.

Possible influence of traces of copper and iron

Copper and iron are well known as oxidation catalysts for butterfat, but the conditions in cheese are not the same as those in milk or butter and the formation of metal-protein complexes may modify the action of the metals. Barnicoat (1937) found that added copper and iron in cheese caused dark discolorations but did not remark on any tallowy flavour even after storage of the cheese for 9 months. Bishop (1961) found that in commercial cheeses showing discoloration the tallowy portions gave lower copper values and higher iron values than the normal portions. Copper and iron contents within the normal range did not appear to be related to the incidence of discoloration. The significance of these findings in relation to tallowy discoloration is obscure.

GENERAL CONCLUSIONS

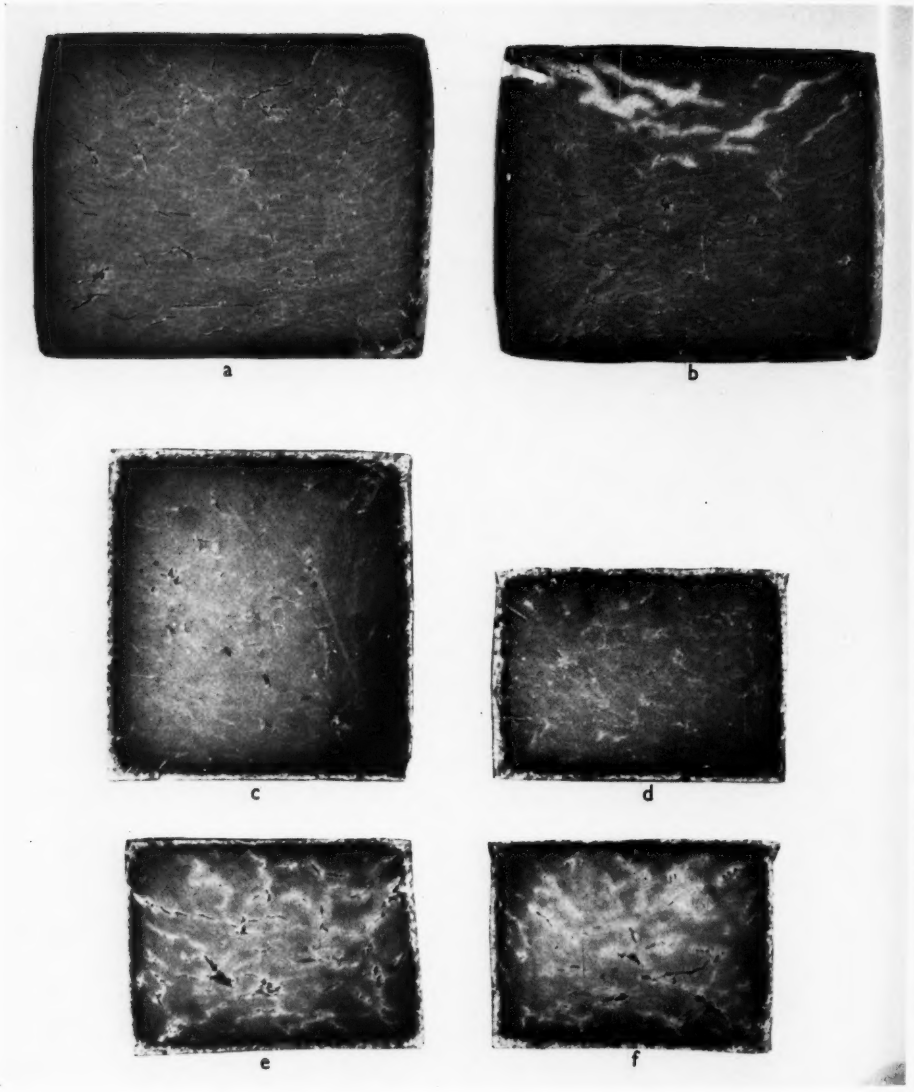
To summarize the present state of knowledge, it is reasonably firmly established that tallowy discoloration in Cheddar cheese consists of an oxidation of the fat and bleaching of the carotene brought about by long contact of the cheese with atmospheric oxygen. The oxidation occurs most readily on moist cheese surfaces such as those presented along internal slits or cracks connecting with the cheese rind or on the outside of cheese blocks wrapped in plastic films which are slightly permeable to oxygen.

The oxidation reaction is hastened by high moisture content, by blood pigments and possibly by low salt concentration. It is delayed by bacterial reducing action which occurs at high storage temperature. Bacteria may operate in two opposing directions. They may promote exposure of the cheese fat to oxygen by the production of gas leading to the formation of slits which, when they reach the surface, admit air to the interior of the cheese. It is also possible that their enzyme systems may catalyse the oxidation of fat. On the other hand, they may by their active growth produce a reducing potential which delays fat oxidation. A commercial cure for the fault of tallowy discoloration appears to lie primarily in producing a cheese without excessive moisture, storing it at a steady, moderate, temperature and avoiding rough handling during any necessary transport. It is also important to exclude oxygen from the surfaces (both external and internal) of the cheese by sealing the surface with a material as impermeable to air as is practicable (e.g. wax or a plastic film). It is possible to inhibit fat oxidation still further by the use of antioxidants. This will be the subject of a later paper.

Thanks are due to many members of the staff of the Institute who helped with advice and criticism over the years in the work described in this paper. We are also grateful to Butland Industries Ltd. for the willing assistance they gave in providing facilities for the examination and disposal of experimental cheeses.

REFERENCES

BARNICOAT, C. R. (1937). *J. Dairy Res.* **8**, 53.
BISHOP, F. (1961). *J. Dairy Res.* **28**, 21.
DOLBY, R. M. (1959). *J. Dairy Res.* **26**, 281.
PONT, E. G. & Rogers, W. P. (1961). *J. Dairy Res.* **28**, 151.
RAMMELL, C. G. (1961). *J. Dairy Res.* **28**, 33.



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(*Facing p.* 149)

EXPLANATION OF PLATE 1

(*a*) Normal cheese 10 months old.

(*b*) Cheese from same vat as (*a*) showing discoloration.

The effects of additions to the cheesemilk

(*c*) Control cheese.

(*d*) Cheese containing sterilized emulsion of discoloured cheese.

(*e*) Cheese containing unsterilized emulsion.

(*f*) Cheese containing mixed culture of bacteria from emulsion.

Printed in Great Britain

Surface oxidation in cold-stored butter: the effects of desiccation and moisture absorption

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(*Received* 3 *January* 1961)

Summary. Unwrapped samples of salted sweet-cream butter were stored in darkness at 1 °C for 6–8 weeks over silica gel, water, isotonic salt solutions, and in free air. Moisture and fat peroxide gradients from the surfaces to the interior of the stored samples were measured in thin sections obtained by a simple macrotome technique. The surface oxidized at the same rate as the rest of the butter and its rate of oxidation was not affected by moderate drying or moisture uptake. With severe surface drying, especially when induced by silica gel, fat oxidation was inhibited, while moisture uptake by the more heavily salted butters promoted surface oxidation.

When butter is held in cold storage the effects of desiccation, oxidation and the absorption of odours can produce a lowering of quality at the surface which at times may assume serious proportions. Pont & Gunnis (1958) and Pont, Dickson & Lattimore (1958) recently reported instances of severe surface deterioration in cold-stored butter characterized by tallowy and oxidized off-flavours, taints absorbed from unsuitable wooden boxes and a lowering of grade scores to 84–85 points compared with 91–92 points in the interior.

Owing to loss of moisture to the paper, to the box and to the atmosphere, some surface desiccation inevitably occurs in parchment-wrapped butter in cold storage. The frequent occurrence of stale, tallowy and oxidized off-flavours in the dry 'primrose' surface layer has led to a common belief in the butter industry that desiccation is a predisposing factor in surface oxidation. This belief is supported by the experimental evidence of Mulder (1948) who found that fat peroxide values at the surface of butter desiccated through being held in a dry atmosphere were higher than those from the surface of butter held under moist conditions. Mulder concluded therefore that as drying of the surface layer was not only unsightly but also promoted oxidation defects, butter in storage should be protected from desiccation. On the other hand Heiss (1960) recently reported that the rind, or dry surface layer formed on butter by loss of moisture, did not oxidize or become stale faster than the interior.

If drying is responsible for surface oxidation, variations in the rate and intensity of drying due to varying atmospheric conditions in cold rooms, different types of containers, or different methods of stacking might well explain, in part at least, the variable incidence of surface deterioration in commercial cold-stored butter observed by Pont *et al.* (1958). The interpretation of Mulder's work, however, is uncertain as his results were based on the examination of butter after 14 days' holding at 18–

24 °C, under which conditions the possibility of interference by bacterial or enzymic activity cannot be disregarded. There is also no reason to expect a causal relationship between desiccation and fat oxidation in butter. This uncertainty led to the present investigation in which a macrotome technique was used to determine moisture and fat oxidation gradients at the surfaces of unwrapped and parchment-wrapped butter stored at near-freezing temperatures over a wide range of relative humidities. The results have shown that desiccation does not predispose butter to surface fat oxidation, but rather that, under extreme conditions of drying, fat oxidation may be inhibited at the surface.

METHODS

(1) *Preparation and treatment of butter samples*

Salted sweet-cream butter was obtained on the day of manufacture in the form of machine-wrapped 1 lb pats taken consecutively from the one churning. The samples were prepared for storage by tempering at 15 °C, removing the wrapper and slicing the pat through the centre parallel with the largest side using a 24 s.w.g. stainless-steel wire held in a hinged rigid frame. This provided an uncontaminated flat surface suitable for sectioning. The butter samples were held for periods varying from 6 to 8 weeks at 1 °C in containers open to the atmosphere or in glass desiccators containing either silica gel, water, or sodium chloride solutions of the same concentration as the salt-in-moisture 'solution' in the butter. A minimum of illumination was used in preparing and sectioning the butter and the samples were stored in total darkness.

(2) *Sectioning of butter*

After storage, sections were cut by hand parallel to the prepared surfaces with a bevelled stainless-steel blade 6·3 cm wide mounted at an angle of 30° on a sliding carriage and operating through a slot 6·4 cm wide and 10 cm long in a stainless-steel plate which was held firmly against the butter (see Fig. 1). The blade was attached to a screw (40 threads per inch) bearing a knurled nut by means of which it could be advanced between individual sections. By working in a cold room at 1 °C it was possible to cut, to a depth of at least 1 cm, successive sections with a thickness of 0·16 mm (corresponding to a half turn of the knurled nut). Usually the first two cuts from the surface were made at the minimum thickness of 0·16 mm and the succeeding cuts with a thickness of 0·32 mm.

(3) *Assay techniques*

With the thinner sections, usually less than 1 g of butter was available for examination. Moisture was determined gravimetrically using a sample of about 0·4 g and heating for 45 min at 105 °C in aluminium dishes. The remainder of the section was used for fat peroxide determinations by the method of Loftus Hills & Thiel (1946); the butter was melted and centrifuged in 50 × 6 mm fermentation tubes and 0·2 ml portions of the supernatant fat were taken with a blood pipette for the peroxide test. Samples for bacterial and yeast and mould counts were taken by scraping a 10 × 1 cm strip of the prepared butter surface to a depth of approximately 1 mm. Total counts were determined on Oxoid milk agar with 5 days' incubation at 30 °C, and yeast and mould counts on acidified wort agar with 7 days' incubation at 25 °C.

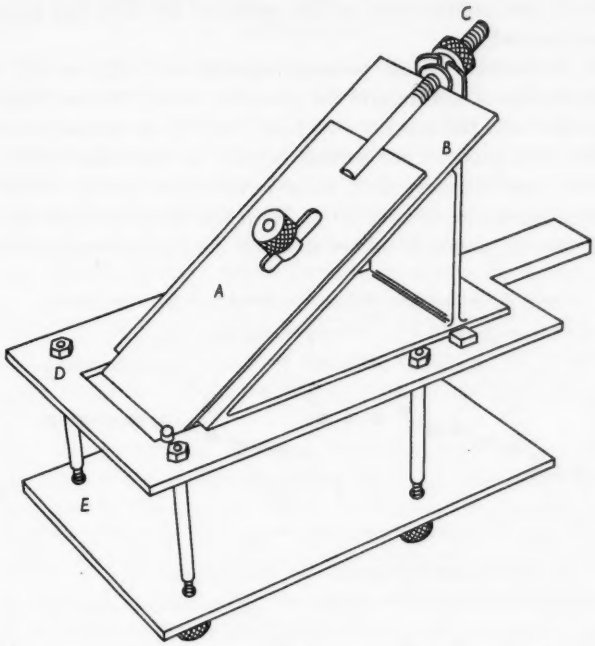

Fig. 1. Perspective drawing of macrotome used for sectioning butter. The bevelled blade *A*, which is 6·3 cm wide, is attached to a sliding carriage *B*. The blade is fed forward by means of the knurled screw *C*. The butter is held firmly against the slotted top-plate *D* by the adjustable base plate *E*.

RESULTS

Five lots of butter were examined at different times with consistent results. Fresh butters before storage had fat peroxide values of 0·02–0·04 m-equiv./kg. After storage for 6–8 weeks values for butter taken from the interior of the pats ranged from 0·2 to 0·5 m-equiv./kg. The spread of values within each batch of butter was slight, being mainly due to small differences in holding times as it was not possible to section and examine more than one or two butters per day.

Butter stored over silica gel rapidly lost water and after 4 weeks the dry surface layer was a little more than 1 mm deep with moisture values of about 2·0 % in the outer 0·16 mm section. Although after a further 4 weeks surface moisture dropped to less than 1·0 % there was very little change in the slope of the moisture gradient at the surface; apparently an equilibrium is established between the rate of diffusion of moisture from the interior and the rate of evaporation from the surface. Drying of this intensity was associated with a progressive fall in peroxide values towards the surface from a depth of about 2 mm (see Fig. 2).

In butters exposed to the air, drying was evident to a depth of about 0·75 mm and the intensity of drying, as judged by the steepness of the moisture gradient towards the surface, was inversely proportional to the salt content. In butters with 1·2 % of salt, surface moistures of 6–8 % were reached and there was usually a slight fall in peroxide values towards the surface. With salt contents of 2·0 % the moisture values

of the outer 0·16 mm section were of the order of 10–12 % but no gradient in fat oxidation was observed.

As expected, butters in vapour-pressure equilibrium with isotonic salt solutions neither gained nor lost moisture and fat peroxide values did not differ significantly between the surface and the interior. In butter held in an atmosphere of 100 % r.h., uptake of water took place in the surface layers. In the more lightly salted butters surface moistures rose to nearly 20 % while in the more heavily salted butters (2 %) they rose in some instances to over 30 %. With the latter samples surface oxidation was promoted, rises in peroxide values towards the surface being evident.

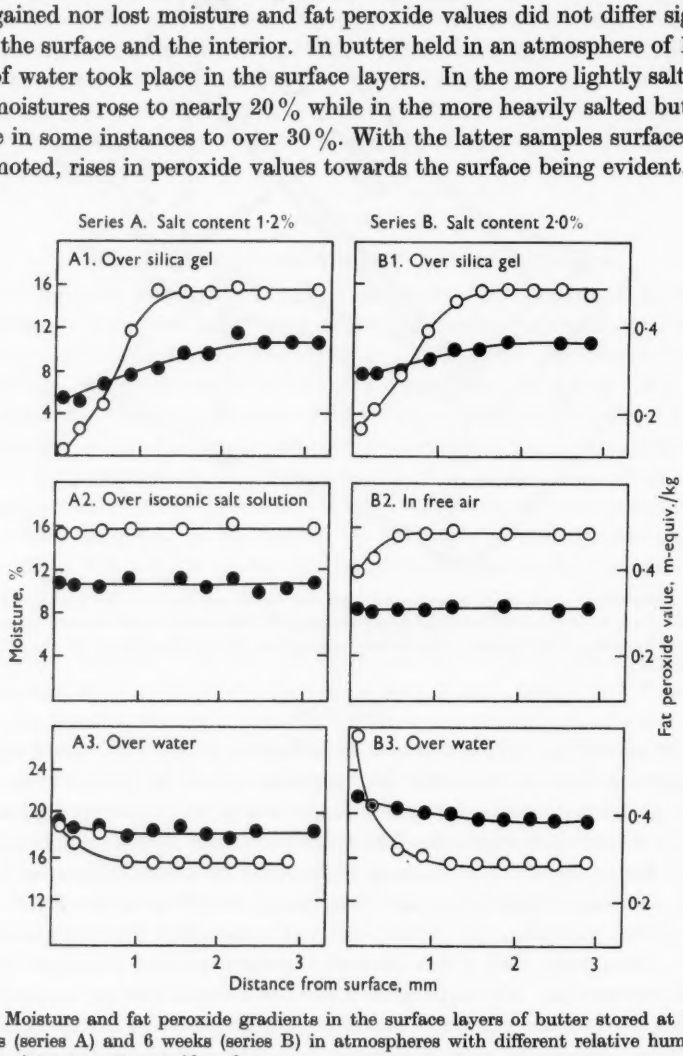

Fig. 2. Moisture and fat peroxide gradients in the surface layers of butter stored at 1 °C for 8 weeks (series A) and 6 weeks (series B) in atmospheres with different relative humidities; ○—○ moisture, ●—● peroxide value.

Total bacterial counts and yeast and mould counts were determined on butters before and after storage. There was no evidence of microbiological activity in the samples during the maximum storage period of 8 weeks. The highest bacterial count after storage was 22000/g. Mould counts generally did not exceed 10/g though in some of the samples exposed to free air, surface counts of over 100/g were obtained; it is believed that these were derived from air-borne spores and not as the result of surface growth.

DISCUSSION

The results have shown that surface drying of butter does not promote fat oxidation but that, in fact, severe desiccation at the surface inhibits oxidation. It may seem surprising at first that availability of atmospheric oxygen does not, *per se*, lead to preferential oxidation at butter surfaces exposed to the air. Many of the proponents of metal foils and other impervious wrapping materials for butter believe that exclusion of air is an essential factor in the control of surface oxidation. Access of air to butter however is not confined to the exterior of the block, there being a very considerable air–butter interface throughout normally worked butter. King (unpublished data) has observed, in locally made butters with air contents of 4%, 40000 air bubbles/cm^3 with an average diameter of 124 μ. The surface area of such a distribution of bubbles would be 19·3 cm^2/cm^3 or 587 ft^2/ft^3. Thus the external 6 ft^2 of air—butter interface at the surface of a 56 lb cube of butter is little more than 1% of the total interface.

The amount of air in butter is, however, normally about 4–5% and if this were not sufficient to sustain fat oxidation throughout the bulk then oxidation would obviously be favoured at the surface. There is abundant evidence however that, in the absence of microbiological activity, air content is not a limiting factor in fat oxidation in butter. In this respect it is sufficient to point to the almost unanimous agreement that vacuum-working, with reduction of air contents from 5% to 1% or less, does not affect the keeping quality of butter in cold storage (Pont, 1960).

The conclusion that neither access of free air, desiccation nor moderate moisture uptake promotes surface fat oxidation in butter narrows the search for causes of surface oxidation occurring in the commercial storage and handling of butter. It follows that preferential oxidation at the surface of butter protected from light, other physical agencies and possibly microbiological activity, can be attributed only to interaction with pro-oxidant substances or compounds derived either from contact with wrapping materials, or less likely perhaps, by absorption from the atmosphere.

REFERENCES

Heiss, R. (1960). *Milchwissenschaft*, **15**, 72.
Loftus Hills, G. & Thiel, C. C. (1946). *J. Dairy Res.* **14**, 340.
Mulder, H. (1948). *Ned. melk- en Zuiveltijdschr.* **2**, 91.
Pont, E. G. (1960). *J. Dairy Res.* **27**, 121.
Pont, E. G., Dickson, J. T. & Lattimore, C. W. (1958). *Aust. J. Dairy Tech.* **13**, 188.
Pont, E. G. & Gunnis, L. F. (1958). *Aust. J. Dairy Tech.* **13**, 68.

Printed in Great Britain

Utilization of urea by milking cows

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(*Received* 12 *January* 1961)

Summary. Friesian cows averaging about 30 lb of milk daily, were used for nitrogen balance experiments of the change-over type, in which 3·0–3·5 lb of two mixtures of molasses and urea were added to basal diets low in protein, but containing a high proportion of starch and lower carbohydrates, and ample energy for the amount of milk produced. The molasses mixtures contained 9–10 % urea, about 2 % H_3PO_4 and were added either alone or with 7 % ethyl alcohol.

In the first experiment, with three cows, the intake of digestible crude protein from the basal diet was about 90 % of the recommended allowance and the utilization of urea nitrogen was poor. In the second experiment, with four cows, the intake of digestible crude protein with the basal diet was about 40 % of the recommended allowance and the utilization of the nitrogen of urea and of additional groundnut meal was almost complete.

Owing to the way in which the experiments developed the addition of alcohol to the mixture of molasses and urea could not be expected in the present work to increase the utilization of the nitrogen of urea even if it does have that effect under other circumstances. When added to the basal diet very low in nitrogen, the nitrogen of both urea and groundnut meal was used mainly to prevent the withdrawal of nitrogen from body reserves, but partly for a small increase in milk yield.

Under the special conditions of this experiment, which were very favourable for the utilization of non-protein nitrogen, the allowances of digestible crude protein recommended by Woodman (1957) for maintenance of cows weighing 1250 lb (0·73 lb) and 1340 lb (0·82 lb) and giving about 30 lb of milk daily (0·6 lb/10 lb milk) did not appear to be excessive.

The use of urea as a substitute for protein in rations for ruminants has long been of interest (see review by Reid, 1953), but since the wartime experiments of Owen, Smith & Wright (1943) no balance studies of the efficiency of the utilization of urea by milking cows have been reported in Great Britain. In the United States commercial claims have led to the investigation of the effect, on food intake and utilization of nitrogen, of including ethyl alcohol in mixtures of molasses and urea given as a dietary supplement. It has been claimed that the alcohol causes reducing conditions to develop in the rumen and that these favour the utilization of non-protein nitrogen. In experiments at this Institute with cows receiving hay or hay with a supplement of molasses and urea, Head (1957) could find no response in pH, Eh or ammonia concentration in the rumen following the administration of 250 ml of alcohol; a small

rise in volatile fatty acid concentration was attributed to fermentation of the alcohol.

The object of the work reported here was to obtain further information on the utilization of urea by milking cows and the effect of additions of alcohol.

EXPERIMENTAL

Mature Friesian cows in milk were used for the experiments. Treatments were arranged according to Latin square designs, the first experiment with three cows, three periods and three treatments and the second with four cows, four periods and four treatments. In both experiments the cows were given control diets low in protein (treatment 1) to which were added a urea molasses mixture (*a*) with alcohol and (*b*) without alcohol (treatments 2 and 3, respectively); in the second experiment sufficient groundnut cake was added to the control diet (treatment 4) to raise the protein intake to 90 % of the requirement.

In Expt. 1 the basal (low-protein) diet consisted daily of 20 lb of hay and a mixture of oats (93 %) and linseed meal (7 %). Since the initial milk yields of the cows were similar, this concentrate was given in equal amounts to all cows and approximately in proportion to their requirement for energy in the early part of the first period. The amounts of concentrates were reduced in the second and third periods as the yield declined. All the cows received 3·5 lb of molasses daily; in treatment 2 this (mix A) contained 9·3 % urea (148 g), 7·1 % ethyl alcohol (113 g) and 2·1 % H_3PO_4 (33 g). The molasses used in treatment 3 (mix B) contained 10·1 % urea (160 g) and 2·3 % H_3PO_4 (36 g).

In Expt. 2 the cows were chosen for their similarity of yield at the same stage of lactation. In the first period the basal (low-protein) diet consisted of 15 lb of hay, 13 lb of flaked maize, 4 lb of dried sugar beet pulp, 3 lb of molasses and 1 lb of starch. In treatment 2 the molasses contained urea, alcohol and H_3PO_4 and in treatment 3 it contained urea and H_3PO_4 in the same percentages as in Expt. 1. In treatment 4 the molasses was replaced by 3 lb of groundnut cake and the starch was reduced to 0·4 lb. In the following periods the amounts of all the foods were reduced by 5·3 %, to conform with declines in lactation without changing the ratios of the foods to each other.

In both experiments the experimental periods consisted of a 2-week preliminary period followed by balance periods of 12 days. Faeces and urine were collected by means of harness and bags (Balch, Bartlett & Johnson, 1951) and samples of the foods, milk and excreta were analysed for nitrogen according to the standard Kjeldahl procedure. Allowance was made for small amounts of food which were left uneaten. In the final period of Expt. 2 the amounts of food remaining uneaten towards the end of the collection period were so large that the period was reduced to 6 days.

In both experiments the intake of urea with treatments 2 and 3 was approximately 1 % of the dietary dry matter and the nitrogen it contained was sufficient, if entirely utilized as crude protein, to supply 38 % in Expt. 1 and 34 % in Expt. 2 of the standard requirement for digestible crude protein with the yields observed with the control diet. In Expt. 2 the urea would thus have been sufficient for about 30 % of the yield found with the diet containing groundnut.

RESULTS

Level of feeding

Unexpected rates of decline in milk yield, or changes in the efficiency of digestion of the crude protein of the diet, could readily spoil the planned allowances of protein. Table 1 shows the adequacy of the diets according to the standards of requirements at present used in this country. The allowances for maintenance given by Woodman (1957) were presumed to be linear from 4·4 lb starch equivalent and 0·39 lb digestible crude protein for an animal weighing 650 lb to about 8·3 lb starch equivalent and 0·85 lb digestible crude protein for a cow weighing 1450 lb. The requirement for the production of milk was taken as 2·5 lb starch equivalent and 0·6 lb digestible crude protein. The intake of starch equivalent was computed by allocating values to the various foods according to the tables given by Woodman, but the intake of digestible crude protein was calculated from the results of the balance periods, ignoring, for this purpose, the urea supplements.

According to the above estimates, the best available to us, the diets in Expt. 1 supplied 121–124% and those in Expt. 2 supplied 129–139% of the standard requirement for starch equivalent. Excluding urea, the diets given with treatments 1–3 supplied, in Expt. 1, 91–95%, and in Expt. 2, 39–43% of the requirement for digestible crude protein; the additional protein supplied by the groundnut meal raised the intake to 90% of the requirement. In these experiments the complete utilization of the energy of the alcohol included in treatment 2 would have raised the daily intake of energy above the values given in Table 1 by more than 0·75 lb starch equivalent; in the experiments of Drori & Loosli (1959) the alcohol supplied, on average, about 0·6 lb starch equivalent.

It is evident, accordingly, that in neither experiment was the intake of net energy by the cows likely to have been limiting. It is also clear that in Expt. 1, as a result of unexpectedly rapid declines in yield, the amount of protein supplied by the basal diet during the balance periods was not especially low. In the second experiment, however, there was every reason to expect the protein intake with the basal diet to be severely limiting, while a high utilization of the extra protein provided as groundnut meal with treatment 4 was to be expected. The amounts of additional nitrogen added as urea in this second experiment were critical since their complete utilization as crude protein would have raised the intake of digestible crude protein to only 68–77% of the standard requirement. By contrast a similar complete utilization of the urea given in Expt. 1 would have raised the intake to about 130% of the requirements.

Utilization of nitrogen added to the diet as urea

The mean amounts of nitrogen given in the diet and in the various additions to the diet and the amounts leaving the body as faeces, urine and milk are summarized in Table 2. The sum of the nitrogen balance and the nitrogen secreted in milk has been used as a measure of the total utilization of nitrogen and is also given in Table 2. Although in both experiments the cows were probably capable of a mean yield of over 30 lb of milk daily the control diets supplied 294 g nitrogen in Expt. 1 but only 157 g in Expt. 2. The additional urea was equivalent to 23–24% of the nitrogen of the

Table 1. *The mean weight of the cows, the milk yield and the requirements and intake of starch equivalent and digestible crude protein*

Expt. no.	Weight of cows, lb	Treatment	Daily milk yield, lb	Daily requirement, lb*		Daily intake, lb†		Daily intake, % of daily requirement	
				Starch equivalent	Digestible crude protein	Starch equivalent	Digestible crude protein	Starch equivalent	Digestible crude protein
1	1343	1. Control	29·6	15·0	2·58	18·6	2·45	124	95
(three cows)		2. Urea + alcohol	29·1	15·0	2·56	18·5	2·41	123	94
		3. Urea	30·6	15·3	2·65	18·5	2·41	121	91
		S.E. of difference	±2·74						
2	1248	1. Control	25·7	13·7	2·31	18·7	0·99	136	43
(four cows)		2. Urea + alcohol	27·8	14·1	2·42	18·6	0·94	132	39
		3. Urea	27·1	14·0	2·35	19·4	0·99	139	42
		4. Groundnut meal	31·9	15·3	2·64	19·7	2·37	129	90
		S.E. of difference	±3·61						

* The requirement of starch equivalent and digestible crude protein were computed according to Woodman (1957), see p. 159.

† The intake of starch equivalent was computed from the values, for individual foods in the diet, given by Woodman. The intake of digestible crude protein was determined in the balance trials and does not include urea.

Table 2. *The mean daily intake, excretion and utilization of nitrogen by the cows*

Expt. no.	Treatment	Intake				Excretion		Utilization		
		Basal diet, g	Supplement			Faeces, g	Urine, g	Milk (*a*), g	Balance (*b*), g	Total (*a*+*b*), g
			Type	Nitrogen, g	% of basal					
1	1. Control	294	—	—	—	116	76	70	32	102
(three	2. Urea + alcohol	297	Mix A	68	23	122	136	73	34	107
cows)	3. Urea	293	Mix B	71	24	118	136	71	39	110
	S.E. of difference	—	—	—	—	± 6·0	± 3·3	± 0·6	± 0·6	± 0·8
2	1. Control	156	—	—	—	85	59	61	−49	12
(four cows)	2. Urea + alcohol	150	Mix A	53	35	82	66	64	−9	55
	3. Urea	152	Mix B	59	39	80	58	62	11	73
	4. Groundnut meal	166	Groundnut meal	87	52	81	78	62	16	94
	S.E. of difference	—	—	—	—	± 7·2	± 12·8	± 6·6	±11·8	± 7·1

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basal diet, given with the urea, in Expt. 1 and 35–39 % in Expt. 2. The groundnut meal supplied nitrogen equivalent to 52 % of the nitrogen of the basal diet.

Using the mean values summarized in Table 2 the fate of the additional nitrogen given as urea or groundnut meal has been calculated and is summarized in Table 3.

Table 3. *The fate of nitrogen added to the basal diet as urea or groundnut meal (g nitrogen as a percentage of g additional nitrogen)**

Expt. no.	Treatment†	Faeces	Urine	Milk (*a*)	Balance (*b*)	Total utilization (*a*+*b*)
1	2 Urea+alcohol	+6·5	+87·1	+4·2	+2·2	+6·4
(three cows)	3 Urea	+3·5	+83·9	+2·4	+10·2	+12·6
2	2 Urea+alcohol	+0·6	+16·1	+11·5	+71·8	+83·3
(four cows)	3 Urea	−4·1	+1·0	+5·2	+97·9	+103·1
	4 Groundnut meal	−10·3	+17·3	+15·9	+77·1	+93·0

* In making these calculations it has been assumed that with each of treatments 2, 3 and 4 the mean percentages of the nitrogen of the basal diets appearing in faeces, urine and milk were as observed with treatment 1.

† The treatment designates the form in which the additional nitrogen was given.

From Tables 2 and 3 it can be seen that little of the additional nitrogen was excreted in the faeces, suggesting that in each instance the urea was largely absorbed from the gut. In Expt. 1, however, 84–87 % was excreted in the urine and only some 6–12 % appeared to be utilized. Under the critical conditions of Expt. 2 only 16 % of the urea appeared in the urine with treatment 2, only 1 % with treatment 3, and only 17 % of the additional nitrogen given as groundnut meal in treatment 4. With the control diet in Expt. 2 the large negative nitrogen balance of −49 g suggests that milk yield was being sustained largely as a result of depletion of body reserves. The evidence suggests that urea in both forms was used both to prevent this withdrawal of protein from the body and to effect a modest improvement in the amount of nitrogen secreted in the milk; this last effect was reflected in the improved yields of milk (Table 1). With treatment 3 the percentage utilization of the urea nitrogen (103 %) appeared to be greater than that of the larger amount of nitrogen given as groundnut (93 %), but the variation between cows makes it unlikely that that difference was significant.

The variation between cows also prevents significance being attached to the slightly larger amounts of nitrogen appearing in the milk with treatment 2 than with treatment 3. In fact, under the conditions of both experiments, there is no evidence that the utilization of urea nitrogen was improved by the addition of alcohol to the diet. It may be argued that improvement could only be expected under conditions where the utilization of urea given without alcohol was incomplete.

DISCUSSION

These experiments have suggested that under very favourable conditions the nitrogen of urea may be almost completely utilized by dairy cows. Favourable conditions were provided by the presence of a large amount of starch and other lower carbohydrates in the rumen and a basal diet which was so low in protein that

it supplied only about 40% of the requirements of the animals. Nitrogen supplied as groundnut meal, in greater amounts than was provided by the urea, was also almost completely utilized. The nitrogen of both urea and groundnut apeared to be used mainly to prevent the withdrawal of nitrogen from body reserves; this withdrawal was necessary in order to continue milk production with the unsupplemented basal diet. However, in Expt. 2 the utilization of urea also permitted a small improvement in milk yield (Table 1).

There was no evidence that the inclusion of alcohol in the diet increased the utilization of urea. This point was not conclusively decided, however, since in Expt. 1 so much protein was provided by the basal diet that it is unlikely that additional nitrogen would have been utilized even if given as protein, whereas in Expt. 2, so little protein was provided by the basal diet that additional urea given without alcohol appeared, unexpectedly, to be completely utilized.

In experiments with growing and fattening beef heifers (Richardson, Smith, Koch & Cox, 1958), growing beef steers (Drori & Loosli, 1959) and growing dairy heifers (Bates, Jacobson, Rust & Seath, 1958), alcohol seems to have had little effect on the utilization of nitrogen or on voluntary food intake.

The opportunity provided by the present nitrogen balance experiments, to define some of the critical conditions for the utilization of non-protein nitrogen, has also emphasized how easily experiments of the feeding-trial type, dealing with the same problem, might be carried out at levels of nitrogen intake which were not critical. The risk must be especially great where diets are devised from published tables of foodstuffs composition. The wide range in apparent utilization of urea, mentioned in the review of Reid (1953), must surely be due largely to differences in the extent to which basal diets contained insufficient protein. The need for balance experiments in parallel with these feeding trials is thus very great.

Feeding standards for nitrogen

In judging the adequacy of the diets used in these experiments the allowances of digestible crude protein recommended by Woodman (1957) have been used. The data collected permit the suitability of these allowances to be examined.

The amount of nitrogen required by a cow is presumably that level of intake which is precisely sufficient, after allowance has been made for losses in the faeces, to provide the nitrogen contained in the milk, the nitrogen required for any body storage normal at that particular age and stage of lactation and the nitrogen required for some minimum level of nitrogen excretion in the urine.

The cows used were probably capable of mean yields of milk of about 31 lb in Expt. 1 and 32 lb in Expt. 2. According to Woodman, their total requirements (Table 1) were therefore about 2·65 lb of digestible crude protein daily (= 192 g digestible nitrogen). In Fig. 1 the total intake of nitrogen, including urea, less the nitrogen in the faeces ('total digestible nitrogen') (Table 2) has been plotted against the nitrogen utilized for milk production and for body storage as demonstrated by the nitrogen balance. It is apparent from Fig. 1 that when the intake of total digestible nitrogen was less than that recommended by Woodman the amount of nitrogen utilized by the animals declined. In the short time of the experimental periods the decline in the nitrogen balance was markedly greater than the decline

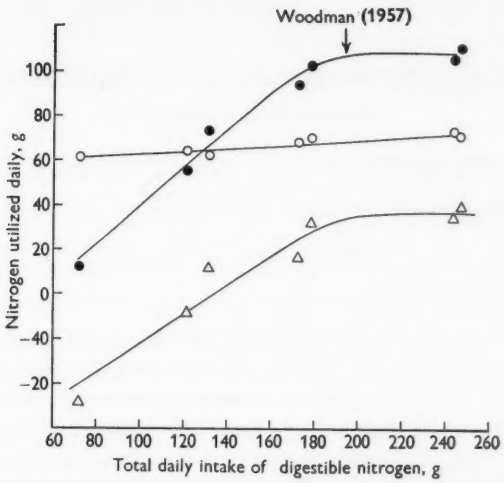

Fig. 1. The effect of the total daily intake of digestible nitrogen on the utilization of nitrogen by milking cows capable of producing at least 31 lb of milk daily. Mean values with each treatment in Expts. 1 and 2 are given from the nitrogen balance (a), △, milk nitrogen (b), ○, and the total utilization of nitrogen ($a+b$), ●. Further details of intake, excretion and utilization are given in Table 2.

in milk nitrogen. Below the allowance recommended by Woodman nitrogen from urea appeared to be utilized as efficiently as nitrogen from other constituents of the diet, but above that allowance additional urea was sparingly utilized. Further evidence of this kind, especially in balances at the end of longer preliminary periods, would show whether the recommended daily allowance of digestible crude protein for milk production in cows should be less than 0·6 lb/10 lb milk produced (Woodman, 1957) as recommended by Bartlett, Foot, Huthnance & Mackintosh (1940). The present results suggest that under these particular conditions 0·6 lb/10 lb milk was satisfactory.

We wish to thank the Directors of the Distillers' Company (Biochemicals) Limited for providing the urea-molasses mixtures used in these experiments. We also acknowledge our debt to Mr V. W. Johnson for much technical assistance.

REFERENCES

Balch, C. C., Bartlett, S. & Johnson, V. W. (1951). *J. agric. Sci.* **41**, 98.
Bartlett, S., Foot, A. S., Huthnance, S. L. & Mackintosh, J. (1940). *J. Dairy Res.* **11**, 121.
Bates, J. P., Jacobson, D. R., Rust, J. W. & Seath, D. M. (1958). *J. Dairy Sci.* **41**, 723.
Drori, D. & Loosli, J. K. (1959). *J. agric. Fd. Chem.* **7**, 50.
Head, M. J. (1957). *Annu. Rep. Nat. Inst. Res. Dairying*, p. 80.
Owen, E. C., Smith, J. A. B. & Wright, N. C. (1943). *Biochem. J.* **37**, 44.
Reid, J. T. (1953). *J. Dairy Sci.* **36**, 955.
Richardson, D., Smith, E. F., Koch, B. A. & Cox, R. F. (1958). *Kansas Agric. exp. Sta. Circ.* 358, p. 65.
Woodman, H. E. (1957). *Bull. Minst. Agric., Lond.*, no. 48.

Printed in Great Britain

J. Dairy Res. (1961), **28**, 165

A viscometric study of the breakdown of casein in milk by rennin and rennet

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(*Received* 18 *January* 1961)

Summary. In the later stages of the reaction between rennin and casein drastic viscometric methods are undesirable, but in the first stages there is a fall in viscosity which may be satisfactorily measured in an Ostwald viscometer. Using fat-free milk, the viscosity at first falls linearly with time. At low rennet concentrations (C_e) this may be said to constitute a zero-order reaction (constant k_0). At higher rennet concentrations and after longer times, the reaction passes to first order (constant k_1). After very long times it doubtless becomes more complex.

The values of k_0 are proportional, over a considerable range, to the milk concentration (C_m), those of k_1 being independent of C_m.

For pure rennin k_0 and k_1 are proportional to C_e, but for commercial rennets they vary as a power (N) of C_e and the value of N appears to measure the rennin purity. The potentialities of the method for assessing rennet activities are also discussed.

If the reduced viscosity (specific viscosity $\div C_m$) of fat-free milks is plotted against C_m, good straight lines are obtained which may be extrapolated to zero concentration to give a reliable value of intrinsic viscosity. The intrinsic viscosity falls progressively during the protein breakdown process but the slope of the curves ('second order term') remains unchanged.

Scott Blair & Burnett (1958*a*) have described a U-tube gelometer for measuring the rate at which the rigidity of coagulated milk gels increases with time. This technique cannot be used until rigidities of about 10 dyn/cm² have been reached. At a slightly earlier stage, when rigidity first makes its appearance, the very small moduli have been measured using a concentric cylinder apparatus as proposed by Hatschek & Jane (1926) but this phase passes so quickly that it seems hardly worth while to make a detailed study of it for the present.

Before there is any rigidity, the milk has ceased to behave as a true fluid, but even before this phase is reached, there is a rise in normal (Newtonian) viscosity due, no doubt, to the formation of aggregates as a first stage in the process of coagulation. During this stage, drastic methods of measuring viscosity, such as capillary, concentric cylinder, or sinker viscometers should not be used, since they destroy the very structure that they are intended to measure.

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Scott Blair & Oosthuizen (1960) have described a rolling sphere viscometer which has the advantage that shearing is very gentle and also that the same material is never sheared twice. While the viscosity is rising, measurements with this instrument give higher values than do those using more drastic methods; but before there is any increase in viscosity, the methods agree.

In the same paper is quoted a number of articles on the changes of viscosity of milk or casein acted on by rennin and similar enzymes, or by commercial rennets. Some authors report a steady rise in viscosity as soon as any change is observed: others find a marked initial fall. All previous workers would appear, however, to have used drastic viscometric methods.

Since the present study is concerned only with the earliest stages, standard Ostwald viscometers, which are more accurate than the rolling sphere viscometer, were used throughout, the usual kinetic energy correction being made in those few experiments in which the fall-time was less than 100 sec.

Viscosities are generally quoted relative to water (η_{rel}) or as specific viscosities ($\eta_{sp} = \eta_{rel} - 1$). However, since other suffixes will be needed in the present paper, 'η' will be written for 'η_{sp}' throughout the text. This quantity, being a ratio of viscosities, is dimensionless. The small differences in density between the samples were ignored, except in the determinations of intrinsic viscosities, i.e. all other viscosities were, strictly speaking, 'kinematic'.

All our experiments showed an initial fall in viscosity, followed by a rise. Without entering into the controversies concerning the chemistry of the casein–rennin reaction, we may regard this initial stage as representing a decomposition of part of the casein, followed in later stages by the formation, first, of aggregates which increase viscosity and later produce viscous anomalies, and finally of a rigid gel structure. The rigidity of this structure is increased by processes of bond formation which will be discussed in later papers.

In the present paper, it is our intention to show that the initial rate of fall of viscosity ($-d\eta/dt$) approximates to very simple equations whose parameters are related to the concentration of milk (C_m) and of enzyme (C_e) and, in the case of commercial rennets, also to the purity of the rennet.

EXPERIMENTAL

For convenience, all the experiments here described were done on reconstituted fat-free low temperature dried milks, using plenty of rennet (one part per 10^4) and working at a temperature of 32 °C. The milk sample was prepared (9 g/100 ml) by making a smooth paste and then stirring for 2 h to facilitate a fine dispersion of milk particles in the water, but avoiding froth formation. The volume adjustment was then made and the sample further allowed to stand in the cold overnight before it was centrifuged at about 5000 *g* for 10 min to remove any 'undissolved' material.

In Fig. 1 is shown a typical curve for the change in specific viscosity with time. Had the rolling sphere viscometer been used, the viscosities in parts 4 and 5 of Fig. 1 would have been appreciably higher.

It is convenient to divide the curve into five parts, though the boundaries are not sharp. From the earliest moment at which it was possible to take a reading, i.e. about

2 min after renneting, the viscosity fell almost linearly with time. With large amounts of rennet it was difficult to draw an asymptote to the curve: with small amounts, there was a longish period during which $-d\eta/dt$ was virtually constant (k_0). As soon as the amount of decomposable casein is appreciably diminished, $-d\eta/dt$ will start to fall. This represents part 2 of the graph. It is reasonable to suppose that the changing slope will be approximately proportional to the amount of decomposable casein still available (so long as the concentration is small) and, also as an approximation, that this is measured by the amount of viscous fall still to come. Were there no subsequent rise of viscosity, this latter would be readily given by $\eta - \eta_{min}$ where η_{min} is the final minimal viscosity. To assume that $(\eta_{max} - \eta_{min})/\eta_{max}$, where η_{max} is the initial value, gives the proportion of casein which is attacked by rennin would be to ignore completely the viscosity of the decomposition products. Nevertheless, this ratio should give a minimal value and this turns out to be about 20 %, which is considerably higher than that generally accepted for the proportion of κ-casein (15 %).

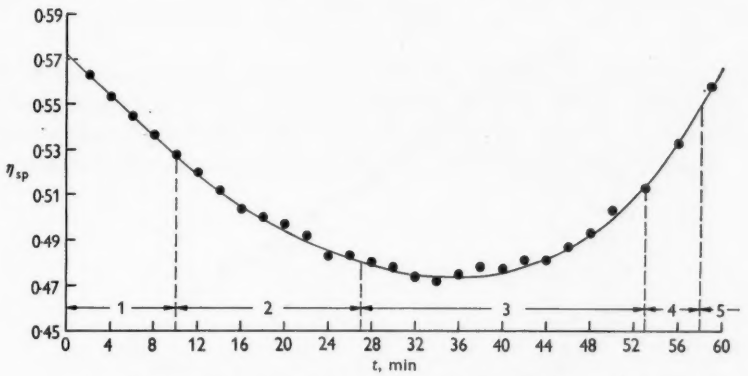

Fig. 1. Changes in specific viscosity of reconstituted fat-free milk (9 g/100 ml) + 100 ppm. rennet at 32 °C and pH 6·7.

Since with milk (as distinct from caseinates) the maximum reduction in fall time is only about 6 sec and since, using this method, errors of timing ~ 0·1 sec are inevitable, accuracies of > 1·5 % can hardly be expected; but an automatic recording viscometer is being made which, it is hoped, will considerably increase accuracy.

The equation for the second part of the curve is:

$$-\frac{d\eta}{dt} = k_1(\eta - \eta_{min}), \tag{1}$$

or

$$kt = \ln\left(\frac{\eta_{max} - \eta_{min}}{\eta - \eta_{min}}\right). \tag{2}$$

This is the equation of a first-order chemical reaction. Scott Blair & Burnett (1958*b*) attempted to apply a similar equation to the rate of setting of the rigid milk curd, but had difficulties through not knowing the ultimate values of the rigidities. Here again, although η_{max} may be fairly accurately determined by a small extrapolation, determining η_{min} is very difficult, since part 3 represents a mixed régime of falling viscosity due to decomposition and of rising viscosity caused by incipient coagulation.

Not very long after the viscosity starts to increase, it also ceases to be independent of shear rate. A horizontal capillary tube sealed at both ends to wider tubes bent through right angles to rise vertically, served as a means for testing this. Though not as accurate as the Ostwald viscometer, this U-shaped viscometer (Scott Blair, 1941) showed an increase in viscosity with the measurable falling head. In Fig. 1, the region where most of the breakdown is presumed to be complete, but where flow is still Newtonian, is marked as part 4, the non-Newtonian region being designated part 5; but it is not possible to draw any sharp lines, especially between 3 and 4.

At low concentrations of casein, η_{min} is hardly affected by coagulation and will be fairly simply related to C_m. Empirically, a plot of η_{min} against $C_m^{1\cdot2}$ was found to be linear. At higher values of C_m, divergences from this straight line may be taken as a measure of the effects of coagulation; but this method is time consuming and not always very accurate. With some casein compounds it is possible to avoid coagulation altogether, by eliminating calcium.

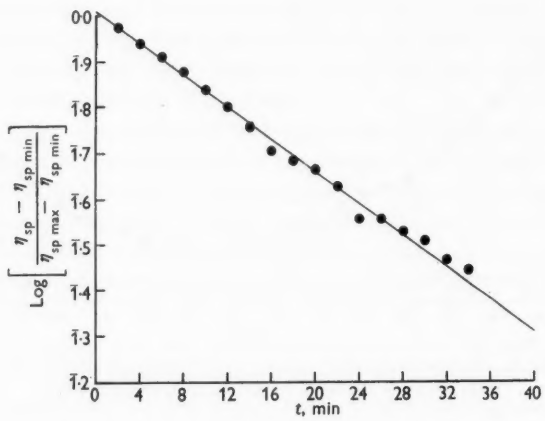

Fig. 2. First-order reaction logarithmic plot for part of the data shown in Fig. 1 ($\eta_{min.}$ was determined by extrapolation of values for diluted samples).

Since the publication of the work by Scott Blair & Burnett (1958*b*), our attention has been drawn to an early paper by Guggenheim (1926) which enables the first-order reaction constant k_1 to be derived without knowing the limits η_{max} and η_{min}. A period of time Δt, representing a fairly high proportion of the whole time of the experiment, is selected. The differences in $\eta(\Delta\eta)$ for each increment Δt over a range of t-values are calculated and $\ln(\Delta\eta)$ is then plotted against t. If the first-order equation holds, these curves should be linear, the slopes being equal to k_1 but with the sign changed. If $\log_{10}$ values are used, the slope is multiplied by 2·30. We have modified Guggenheim's original proposal in that we draw, by eye, the best possible smooth curve through the experimental points, reading the values of η from this curve and not taking the experimental values themselves. Fig. 2 shows a typical curve for reconstituted milk. We have obtained some hundreds of curves of this kind.

Assuming the value of k_1 to be correct and that equation 2 above holds, values of η_{max} and η_{min} may be calculated directly from the Guggenheim curves. The first few points were often slightly off the line and, towards the end of the experiment, there was often evidence of a regular deviation such as is seen in Fig. 2. This latter phenomenon is very usual in many enzyme reactions.

We are unaware of any earlier attempts to fit kinetic equations to viscometric data for casein and in only one case have we been able to fit the first order equation to published data. Data given by Söhngen, Wieringa & Pasveer (1937) would appear to give quite good curves, similar to that shown in Fig. 2.

In equation 1, when $t = 0$, $-\frac{d\eta}{dt} = k_0$ (the asymptote) and we have

$$-\frac{d\eta}{dt} = k_0 = k_1(\eta_{max} - \eta_{min}),$$

or

$$\frac{k_0}{k_1} = \eta_{max} - \eta_{min}. \tag{3}$$

In view of the assumptions given above, this implies that k_1, for any given milk, should be independent of C_m, and k_0 proportional to it, over a reasonable range. Fig. 3 shows that this is indeed the case. At higher values of C_m, k_0 will eventually become constant, but experimental difficulties arise at high concentrations.

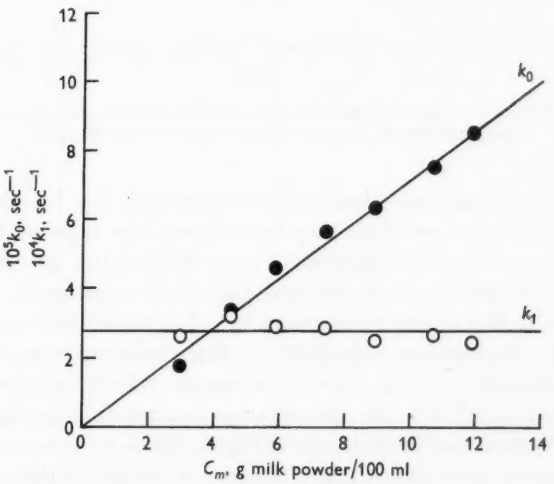

Fig. 3. Variation of kinetic constants with milk concentration. (The same conditions as Figs. 1 and 2.)

When quite small quantities of rennet were used, most of the experimental curves gave a constant rate of fall for viscosity. As rennet concentration was gradually reduced, it is impossible to say at what point this should be regarded as a separate zero-order reaction, since the earlier part of a logarithmic curve is indeed very nearly

linear. Nevertheless, at really low rennet concentrations, there seems little doubt that, within the limits of experimental error, the zero order equation holds

$$-\frac{d\eta}{dt} = k_0. \tag{4}$$

As early as 1902, A. J. Brown (1902) found that the action of invertase on sucrose does not follow the law of mass-action, as he put it, or, as we should say today, is of zero order. In the same year, H. T. Brown & Glendinning (1902) found similar behaviour for the hydrolysis of starch.

One of the many curves which we should regard as illustrating a zero-order reaction is shown in Fig. 4.

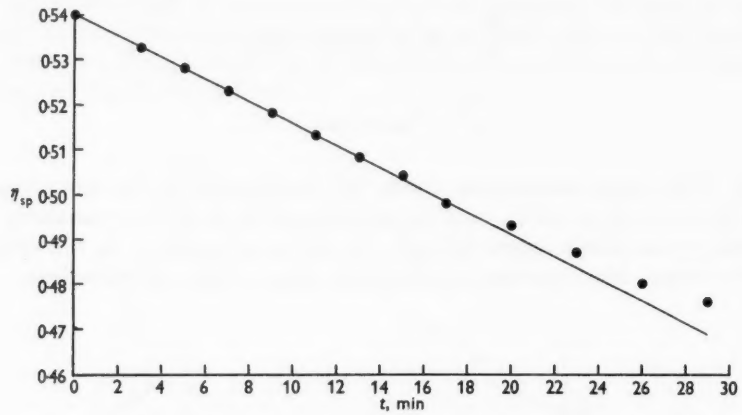

Fig. 4. First part of specific viscosity–time curve illustrating zero-order reaction. (Same conditions as earlier figures except rennet 50 ppm.)

So far as we can tell, at concentrations of enzyme such that (in a long experiment) both zero- and first-order reactions may be followed, the value of k_0 for the former does not differ from that of the asymptotic value of the latter as calculated from the Guggenheim plot of the data from the later part of the experiment. Further experiments were done to find out how the values k_0 and k_1 varied with changes in enzyme concentration (C_e) for the same values of C_m. For a pure enzyme, one would expect a direct proportionality. Using crystalline rennin (kindly supplied by Dr N. J. Berridge) this was found to be the case, whereas commercial rennets showed a curvilinear relation. Typical curves are shown in Fig. 5. Since we cannot compare directly the concentrations of pure rennin and a commercial rennet, whose rennin content is unknown, values of C_e in the figure are given in arbitrary units. It is conveniently found that such curves become linear when plotted with double logarithms. So we may write

$$k_1 = AC_e^N, \tag{5}$$

where A and N are constants.

For pure rennin, $N = 1$ and it seems likely that the divergence of N from unity will give a measure of the impurity (as distinct from activity) of commercial rennets.

It must be admitted, however, that preliminary experiments in which rennin was mixed with other substances separated from rennet, and with pure pepsin, did not show any simple quantitative relationships with the value of N. Further experiments are in progress.

We have seen that an empirical relation was obtained between η_{min} and C_m for low concentrations. This suggested experiments to find what equation really fitted the viscosity-concentration relations of fat-free milk. A few years ago, in this laboratory, Sterling (1958) showed that the alkaline caseinates as prepared by him did not follow any very simple equation. The usual plot of η_{sp}/C_m against C_m did not give straight lines.

Our own experiments on fat-free dried milks, however, give straight lines over a considerable range of concentration. One such curve is seen in Fig. 6. (Each point represents an average of about four viscosity determinations.)

These follow the well-known equation

$$\eta_{sp} = [\eta]C_m + \theta C_m^2, \tag{6}$$

where $[\eta]$ is intrinsic viscosity. The first proposal for an expansion of Einstein's equation of this type is probably due to Guth (1936). As the viscosity falls under the influence of 1·0 parts per 10^4 of rennet, so $[\eta]$ falls from about 0·0385 dl/g to about 0·0347 dl/g during the interval between 2 and 12 min after renneting. The second-term constant θ remains unchanged.

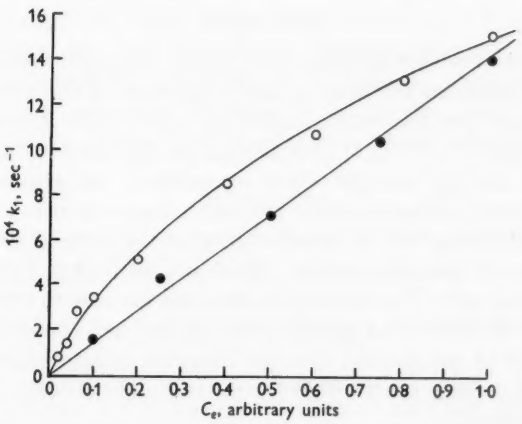

Fig. 5. Variations of first-order chemical reaction constants with enzyme concentration.
●—●, rennin; ○—○ rennet.

Finally, experiments were done to see whether the fall in viscosity is much affected by calcium, sodium and hydrogen ions. Unlike the subsequent coagulation, calcium has little effect on the first two parts of the viscosity curve. Sodium chloride diminishes k_1 but only when added in relatively large quantities ($> 0{\cdot}3\,\%$). The pH is important, as is already well known, but this is easily controlled and there is, in any case, only a rather limited range of pH over which these experiments can be effectively done.

With the help of Dr S. J. Rowland and Mr A. Wagstaff, measurements were made of the increase of non-protein nitrogen, parallel to a viscosity experiment. The former curve can also be expressed as a first-order reaction, as Nitschmann & Bohren (1955) have shown. In our experiment, k had a value of $8{\cdot}7 \times 10^{-4}$ sec^{-1} as compared with $6{\cdot}9 \times 10^{-4}$ sec^{-1} for k_1 from the viscosity fall.

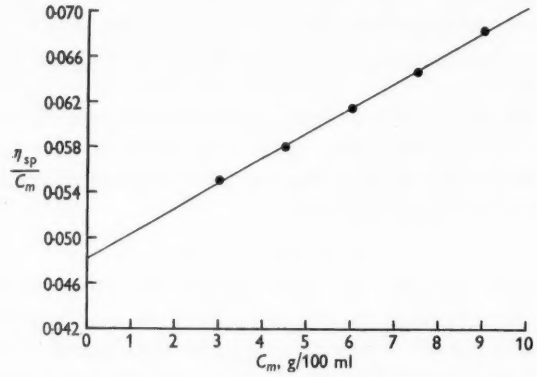

Fig. 6. Intrinsic viscosity [η] curve for fat-free dried milk.

DISCUSSION

Nitschmann and a number of workers in France, especially Garnier, have studied the breakdown of fractions of casein by rennet in terms of the rate of production of non-protein nitrogen (see Nitschmann & Bohren, 1955; Alais, Mocquot, Nitschmann & Zahler, 1953; Garnier, 1957*a*, *b*) This method has the great advantage that all the complications of clotting, brought about by calcium, are avoided. On the other hand, such analytical determinations are much more trouble than are viscosity measurements and it may well be possible to refine the latter so as to give considerably higher accuracy than the former. Moreover, as Irving, Fruton & Bergmann (1941) have pointed out: 'The assumption that the increase in non-protein nitrogen is proportional to the number of peptide bonds hydrolysed appears to have no sound basis. The amount of non-protein nitrogen liberated is determined by the position of the hydrolysed peptide bond within the protein molecule and also by the nature of the split products.'

In the present paper, we have discussed work on fat-free milk only. Unless soluble calcium is removed (and we have not yet found any satisfactory method for doing this) it is hard to determine the true minimal viscosity because of incipient coagulation. The use of Guggenheim's method might, at first sight, appear to obviate this difficulty; but, since it is a purely mathematical device, it cannot overcome a difficulty that is ultimately experimental. For adequate accuracy, fairly large values of Δt must be used and this introduces the anomalies at the end (and to a lesser extent at the beginning) of the process into the calculation of data which themselves fall within the central and regular part of the curve. It should also be pointed out that the values of η_{max} and η_{min} calculated from the Guggenheim plot rest on the assump-

tion that the best straight line drawn through the $\ln(\Delta\eta):t$ points really does represent the course of a first-order reaction within the specified range of time. They do not represent experimental values of the specific viscosity at the start and at the end of the process of protein breakdown.

Experiments similar to those here described are now in progress using sodium caseinates, where this difficulty does not arise. On the other hand, the experiments by Sterling (1958) did not suggest that his sodium caseinates showed at all simple viscosity–concentration relations. We are now investigating other methods of preparing caseinates on which we hope to report in due course.

It seems likely that viscometry should provide a useful means of assessing the activity (as k_0 or k_1) and the purity (as N) of commercial rennets if a suitable reproducible form of casein can be found and if viscometric experimental errors can be further reduced. It is not yet possible to say whether values of k_0 at low concentrations of rennet or of k_1 at higher concentrations serve better as a measure of activity. The former has the advantage that equation 4 has only two parameters (in its integrated form) and all that is required is to draw a straight line through the experimental points, whereas equation 2 is a three parameter equation, requiring a smooth curve to be drawn by eye. On the other hand, k_1 is measured at enzyme concentrations more near those used in cheese making and the first order reaction probably represents a truer measure of enzyme activity if only it can be measured with sufficient accuracy.

Our best thanks are due to many of our colleagues at the N.I.R.D. too numerous to mention individually, who have given much useful advice; also especially to Prof. A. Katchalsky of the Weizmann Institute of Science, Israel, for many helpful discussions; and to Monsieur G. Mocquot, of the Station Centrale de Recherches Laitières, France, for proposing this line of work to us and for his continued interest and advice.

REFERENCES

Alais, C., Mocquot, G., Nitschmann, H. & Zahler, P. (1953). *Helv. chim. acta*, **36**, 1955.
Brown, A. J. (1902). *J. chem. Soc.* **81**, 373.
Brown, H. T. & Glendinning, T. A. (1902). *J. chem. Soc.* **81**, 388.
Garnier, J. (1957*a*). *Ann. Technol.* **3**, 245.
Garnier, J. (1957*b*). *Proc. Int. Symp. Enzyme Chem.*, p. 524. Tokyo and Kyoto.
Guggenheim, E. A. (1926). *Phil. Mag.* **2** (7th ser.), 538.
Guth, E. (1936). *Kolloidzschr.* **74**, 147.
Hatschek, E. & Jane, R. S. (1926). *Kolloidzschr.* **39**, 300.
Irving, G. W., Fruton, J. S. & Bergmann, M. (1941). *J. biol. Chem.* **188**, 231.
Nitschmann, H. & Bohren, H. U. (1955). *Helv. chim. acta*, **38**, 1953.
Scott Blair, G. W. (1941). *Biochem. J.* **35**, 267.
Scott Blair, G. W. & Burnett, J. (1958*a*). *J. Dairy Res.* **25**, 298.
Scott Blair, G. W. & Burnett, J. (1958*b*). *J. Dairy Res.* **25**, 457.
Scott Blair, G. W. & Oosthuizen, J. C. (1960). *Brit. J. appl. Phys.* **11**, 332.
Söhngen, N. L., Wieringa, K. T. & Pasveer, A. (1937). *Rec. Trav. Chim. Pays-bas*, **56**, 280.
Sterling, C. (1958). *J. Dairy Res.* **25**, 307.

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A note on the carbon dioxide requirement of *Streptococcus lactis*, strain ML_3

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(*Received* 30 *January* 1961)

It is now well recognized that carbon dioxide is involved in the synthesis of fatty acids, aspartic acid, purines and pyrimidines. In the course of nutritional and metabolic studies of the lactic acid streptococci it became clear that the varying CO_2 content of the synthetic amino acid medium employed (mainly according to McLeod & Snell, 1950) could be critical for the determination of some growth requirements. When cultures of a representative selection of *Streptococcus lactis* and *Str. cremoris* strains were incubated anaerobically under nitrogen or carbon dioxide the results tended to be erratic. This was thought to be caused by the difficulty of removing dissolved gases from a liquid medium. A more effective way of removal of the gases appeared to be the continued agitation of the medium with CO_2 free N_2, the inlet tube dipping below the surface of the medium in about 1 in. diameter culture tubes. Under these conditions growth was completely prevented.

Whitehead, Jones & Robertson (1958) have reported that lactic acid streptococci showed a prolonged lag period in milk when the CO_2 was continuously swept away by a stream of CO_2 free N_2. Furthermore, 0·5 % yeast extract added to milk substituted for the CO_2 requirement.

This note presents evidence that *Str. lactis* ML_3 (which showed the shortest lag period without CO_2 according to Whitehead *et al.* 1955) has an absolute requirement for CO_2 both in the synthetic amino acid medium and in milk with or without the addition of yeast extract.

The medium (10 ml) in the special tubes was swept at 30 °C with either CO_2 free N_2 or N_2 and 5 % CO_2 for 1 h before inoculation. The CO_2 free N_2 was passed through a sintered glass disc in a wash bottle containing N-NaOH before use. The media were then inoculated with a washed suspension of cells grown in synthetic medium to give finally 1×10^7 cells/ml. The cultures were incubated at 30 °C with a continuous flow of sweeping gas at a rate of about 20 ml/min.

Additional control cultures in cotton wool plugged tubes were incubated under aerobic conditions.

Table 1 shows that ML_3 failed to grow in any of the media under CO_2 free N_2, but grew normally in both types of control cultures. Prolonged incubation up to 64 h with continuous sweeping failed to produce any growth.

As the homo-fermentative lactic acid streptococci are known to produce small amounts of CO_2 we investigated whether ML_3 was capable of initiating growth in CO_2 free media. For this purpose the media were agitated with CO_2 free N_2 for 1 h before inoculation and for 30 min after. The gas supply was then cut off and the gas outlet sealed.

Table 1. *Inhibition of growth by continuous agitation of media with CO_2 free N_2 after 18 h incubation at 30°C*

Medium	Swept with CO_2 free N_2	Swept with N_2 + 5% CO_2	Aerobic cultivation
Amino acid medium*	100	9	21
Amino acid medium + 0·5% yeast extract	100	–	6
Litmus milk	NR	×	×
Litmus milk + 0·5% yeast extract	NR	×	×

* Percentage transmission, 100 = no growth; NR = not reduced; × = clotted.

Table 2. *Growth in CO_2 free media without continuous agitation*

Medium	Swept with CO_2 free N_2	Sealed after agitation for 1 h before and 30 min after inoculation	Aerobic	Time of incubation, h
Amino acid medium*	100	100	—	18
	100	16	—	48
Litmus milk	NR	×	×	18

* Percentage transmission, 100 = no growth; NR = not reduced; × = clotted.

Table 2 shows that strain ML_3 is capable of growing without an outside supply of CO_2 in both synthetic media and in milk, after a prolonged lag phase.

We could not confirm under our conditions of cultivation of ML_3 that the absence of CO_2 caused only a prolonged lag phase, but found that this organism had an absolute CO_2 requirement in both the synthetic medium and in milk, which could not be replaced by yeast extract. It appears, however, that sufficient CO_2 is produced metabolically to initiate growth if the culture is not swept continuously with CO_2 free gas.

REFERENCES

McLEOD, R. H. & SNELL, E. E. (1950). *J. Bact.* **59**, 783.
WHITEHEAD, H. R., JONES, P. A. & ROBERTSON, P. S. (1958). *J. Dairy Res.* **25**, 24.

Printed in Great Britain

The effect of hydrogen peroxide on the nutritive value of milk

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(*Received* 23 *February* 1961)

Summary. Treatment of milk with 0·05 % (w/v) H_2O_2 for 8 h at 24 °C had no significant effect on the concentrations of seven of the B-complex vitamins or of the fat-soluble vitamins A and E or of carotene. Rat tests showed that the nutritive value of the milk proteins was slightly reduced. This finding was confirmed by microbiological tests with *Streptococcus zymogenes*, which also showed that the loss was probably connected with that of methionine. The concentration of H_2O_2 used was effective in controlling bacterial growth during the incubation period, even in a sample of milk with a high initial bacterial count.

Hydrogen peroxide has been used as a preservative in milk for many years. Recently it has come under consideration by the Food and Agriculture Organization of the United Nations (F.A.O., 1957), which concluded broadly that the use of any preservative in milk is undesirable and should be adopted only in exceptional circumstances, such as obtain in countries which do not have a highly developed milk handling system. The report further emphasizes that H_2O_2 merely improves the keeping quality of the milk and should not be substituted for pasteurization since, at the recommended concentrations of 0·01 to 0·08 % (w/v), certain types of pathogenic organisms are not destroyed.

The value of H_2O_2 in restricting the growth of bacteria in milk, and other aspects of its use as a milk preservative, have been reviewed by Lück (1956). In particular, increasing use is being found for H_2O_2-treated milk in the cheesemaking industry (Roundy, 1958).

Although much work has been done on the effect of H_2O_2 on the microflora of milk, there have been few comprehensive reports of its effect on nutritive value. We therefore investigated the effect of H_2O_2 on the nutritive value of the proteins, vitamin content and bacterial flora of a sample of bulk milk incubated for 8 h at 24 °C. Two subsamples of milk were studied, one with a high bacterial count (deliberately contaminated) and the other with a low count (clean), each treated with 0·05 % (w/v) of H_2O_2. From the range of concentrations (0·02–0·12 %, w/v) used by

other investigators (Lück, 1956), the level of 0·05 % (w/v) H_2O_2 was chosen as being suitable for controlling the bacterial flora of milk during transport from farm to factory in hot countries.

EXPERIMENTAL AND RESULTS

Treatment of the milk. A 100 lb sample of bulk milk (bulk 1) was obtained uncooled, directly from the cowshed as soon as possible after milking. It was mixed well and divided into two portions, one being placed in a sterile can and the other in a contaminated can prepared as described by Cousins, Hoy & Clegg (1960). This method was selected as simulating the type of contamination of milk that occurs at milking if poorly cleaned and sterilized equipment is used. The contents of each can were again divided and this time placed in sterile stainless-steel containers. H_2O_2 (30 %, w/v) was added to one portion of clean and to one of contaminated milk, to give a final concentration of 0·05 % (w/v). Following the recommendation of Rosell, Dagá & Fernandez (1959), we added the H_2O_2 within 1 h of milking, when the natural catalase content of the milk is at its lowest. The H_2O_2-treated milks and the corresponding untreated control milks were then incubated at 24 °C for 8 h. Catalase (L. Light and Co., Colnbrook, England) was then added to the H_2O_2-treated milks at the rate of 19 mg/25 lb. The destruction of H_2O_2 by it was checked by the potassium-iodide test and found to be slow, so that it was necessary to add a further 10 mg of catalase/25 lb. Consequently 1 h elapsed before the destruction of H_2O_2 was complete. All four milk samples were immediately transferred to the deep-freeze and subsequently freeze-dried to facilitate handling in the biological tests. During freeze-drying part of the sample of clean milk treated with H_2O_2 was overheated owing to temporary difficulties with heat transfer, and it was considered inadvisable to use it in the biological tests. A second bulk of clean milk (bulk 2) was therefore treated with H_2O_2, as above, for these tests.

Bacteriological tests. Samples of the clean and contaminated milks from bulk 1 were taken at the beginning of the experiment and again after 8 hours' incubation at 24 °C in the presence and absence of H_2O_2. The colony counts of these samples were determined using Yeastrel milk agar (Y.M.A.), replicate plates being incubated at 37 °C for 2 days and at 30 °C for 3 days. Presumptive coli-aerogenes tests in MacConkey broth (at 30 °C) and titratable acidity determinations were also made. The results of these tests (Tables 1 and 2) show that H_2O_2 effectively controlled the growth of bacteria in the milk during the experimental period.

Table 1. *Results of bacteriological examination of untreated and* H_2O_2*-treated* (0·05 %, *w/v*) *milks from bulk* 1 *before and after incubation at* 24 °C *for* 8 *h*

	H_2O_2 addition	Hours of incubation	Colony counts/ml in Y.M.A. at 37 °C	Colony counts/ml in Y.M.A. at 30 °C	Presumptive coli-aerogenes test
Clean milk	−	0	1×10^3	$1{\cdot}5 \times 10^3$	− 1 ml
	−	8	$1{\cdot}5 \times 10^6$	$1{\cdot}5 \times 10^6$	$+10^{-2}$ ml
	+	8	4	5	− 1 ml
Contaminated milk	−	0	1×10^5	$5{\cdot}5 \times 10^5$	$+10^{-4}$* ml
	−	8	8×10^9	12×10^9	$+10^{-7}$* ml
	+	8	1×10^3	$2{\cdot}5 \times 10^3$	$+10^{-2}$ ml

* Higher dilutions were not tested.

Table 2. *Acidity expressed as percentage of lactic acid developed in untreated and* H_2O_2*-treated* (0·05 %, *w*/*v*) *milks from bulk* 1 *during* 8 *hours' incubation at* 24 °C

Hours of incubation	Clean milk		Contaminated milk	
	Untreated	H_2O_2-treated	Untreated	H_2O_2-treated
2	0·15	0·15	0·15	0·15
4	0·15	0·15	0·19	0·15
6·5	0·15	0·15	0·20	0·15
7·5	0·16	0·15	0·20	0·16
9	0·16	0·15	0·22	0·16

The predominant micro-organism (comprising 30–50 % of the total count) in the fresh milk was found to be a coagulase positive staphylococcus. The milk deliberately placed in a contaminated can showed an increased colony count with a predominant flora of Gram negative rods, which included the coli-aerogenes group. Titratable acidity determinations showed the presence of vigorous milk-souring organisms (Table 2).

After treatment with H_2O_2 a few aerobic spore formers only were isolated from the clean milk, whereas with the contaminated milk the residual count was higher, a coagulase negative micrococcus being the predominant organism. The H_2O_2 treatment was effective in controlling acid development in the milks during the experimental period. It also effectively reduced the numbers of Gram negative rods in the contaminated milks, but failed to destroy completely organisms of the coli-aerogenes group, which were present in samples of 0·01 ml at the end of the treatment period. The coagulase positive staphylococci were not isolated from the treated milks but the tests used were not sensitive enough to reveal their survival in small numbers.

Measurement of the water soluble vitamins. Biotin, nicotinic acid, Ca pantothenate, riboflavin, pyridoxal (vitamin B_6) and vitamin B_{12} in the freeze-dried milks, were measured by the microbiological methods described by Gregory, Ford & Kon (1958). Thiamine was measured with *Lactobacillus fermenti* 36 by the method of Sarett & Cheldelin (1944). Table 3 shows that H_2O_2 had no significant effect on the levels of biotin, nicotinic acid, Ca pantothenate, riboflavin, thiamine or pyridoxal in the milks. Only with vitamin B_{12} was a loss of some 15 % observed, but the number of samples studied was insufficient to establish whether this was a significant loss. No vitamin C determinations were done as the destructive effect of H_2O_2 on this vitamin is well established (see Lück, 1956).

Measurement of the fat soluble vitamins. Vitamin A, carotene and the biologically inactive xanthophylls were measured as described by Kon & Thompson (1957). α-Tocopherol was determined by the method recommended by the Analytical Methods Committee (1959). It can be seen from Table 3 that treatment with H_2O_2 caused no loss of these factors.

Nutritive value of the proteins. The biological value and true digestibility of the proteins were determined on groups of twelve rats, at an 8 % level of protein intake, by the method of Mitchell (Mitchell, 1923–24; Mitchell & Carman, 1926) as used in this laboratory (Henry, Kon & Watson, 1937; Henry, Kon, Lea & White, 1947–48; Henry & Kon, 1956). The freeze-dried samples prepared from bulk 1 were used, with the exception of the H_2O_2-treated clean milk which was replaced by the corresponding

one prepared from bulk 2. The untreated milk from bulk 2 was not tested as it was not possible, technically, to study five samples in a single test. There was little likelihood of any marked difference in the biological value of the proteins of the two samples of bulk milk, and it was considered important to have a direct comparison between the untreated clean and the two contaminated milks from the same bulk.

Table 3. *The vitamin content of clean and contaminated milks incubated at* 24 °C *for* 8 *h in the presence and absence of* 0·05 % (*w*/*v*) H_2O_2. (*Mean values from two assays*)

	Bulk 1				Bulk 2	
	Clean		Contaminated		Clean	
Vitamin	Untreated	H_2O_2-* treated	Untreated	H_2O_2-treated	Untreated	H_2O_2-treated
Biotin (μg/g freeze-dried milk)	0·14	0·16	0·15	0·15	0·16	0·14
Nicotinic acid (μg/g freeze-dried milk)	3·83	3·62	4·25	4·68	5·45	5·83
Ca pantothenate (μg/g freeze-dried milk)	15·7	15·7	15·0	15·4	23·6	25·8
Riboflavin (μg/g freeze-dried milk)	8·6	7·7	10·1	8·8	9·1	10·7
Thiamine HCl (μg/g freeze-dried milk)	2·65	2·80	2·98	2·83	2·97	2·98
Pyridoxal (μg/g freeze-dried milk)	1·90	2·16	1·66	2·05	2·56	2·13
Vitamin B_{12} (mμg/g freeze-dried milk)	35	30	40	35	36	30
Vitamin A (μg/g fat)	6·3	6·8	6·9	6·4	7·1	7·4
Carotene (μg/g fat)	4·2	4·0	4·6	3·8	6·2	6·7
Xanthophyll (μg/g fat)	0·6	0·6	0·6	0·5	1·4	1·5
α-Tocopherol (μg/g fat)	12·6	12·7	13·2	9·8	13·7	15·4

* Overheated during freeze-drying, see p. 178.

Table 4. *The nutritive value of the proteins of clean and contaminated milks incubated at* 24 °C *for* 8 *h in the presence and absence of* 0·05 % (*w*/*v*) H_2O_2

	Bulk 1			Bulk 2	
		Contaminated		Clean	
	Clean, untreated	Untreated	H_2O_2-treated	Untreated	H_2O_2-treated
Biological tests with groups of twelve rats					
Biological value of the proteins	90·5	88·8	87·0	—	87·9
S.E.M.*	±0·88	±0·88	±0·88		±0·88
True digestibility of the proteins	94·8	93·9	92·4	—	91·9
S.E.M.*	±0·61	±0·61	±0·61		±0·61
Total nutritive value (biological value × true digestibility ÷ 100)	85·8	83·4	80·5		80·8
Microbiological tests with Streptococcus zymogenes					
Relative nutritive value of the proteins (casein = 100)	—	—	—	115	108
Total methionine (g/16 g N)	—	—	—	3·12	2·90
'Available' methionine (g/16 g N)	—	—	—	2·94	2·50

* Standard error of the mean.

The nutritive value, and the total and 'available' methionine in the two samples prepared from the second bulk of milk were measured microbiologically with *Streptococcus zymogenes* NCDO 592 as described by Ford (1960).

Compared with those of the untreated clean milk, both the biological value and the true digestibility of the proteins of the two H_2O_2-treated samples were slightly, but significantly ($P < 0{\cdot}05$), reduced (Table 4). The resulting loss in total nutritive value (biological value × true digestibility ÷ 100) was about 6 %. This loss was confirmed for clean milk in the microbiological tests on the bulk 2 samples. These tests also showed that treatment with H_2O_2 reduced both the total and 'available' methionine and indicated that the impairment of nutritive value was probably due to a specific effect on this acid, which is known to be unstable in the presence of H_2O_2.

DISCUSSION

Our results show that a concentration of 0·05 % (w/v) H_2O_2 in contact with milk over a period of 8 h at 24 °C (75 °F) had no detrimental effect on the vitamins measured and only slightly reduced the nutritive value of the proteins. Furthermore, this treatment with H_2O_2 reduced the bacterial count of the milk when it was deliberately contaminated with a large number of micro-organisms commonly found in dirty milk cans.

The bactericidal efficiency of H_2O_2 depends on its concentration and on the temperature and duration of the treatment. Although prolonged treatment and high concentrations of H_2O_2 bring about changes in the milk proteins, they have no great effect on the vitamins other than vitamin C which is rapidly inactivated (Lück, 1956). Lück & Schillinger (1958) found no loss of thiamine, riboflavin or vitamin B_6 in milk treated with 0·07 % (w/v) H_2O_2 for 30 min at 51 °C, or of β-carotene and vitamins A and E in milk treated with either 0·075 % (w/v) H_2O_2 for 30 min at 51 °C or with 0·3 % (w/v) H_2O_2 at 30 °C for 24h. Similarly Nambudripad, Laxminarayana & Iya (1952) found that riboflavin, nicotinic acid, thiamine and vitamin B_{12} were unaffected by treatment of milk with 0·03 % (w/v) H_2O_2 before pasteurization at 63 °C for 30 min.

Teply, Derse & Price (1958) used higher concentrations of peroxide and found that treatment for a short time (10 min) at a high temperature (49 °C) with 0·1, 0·2 or 0·5 % (w/v) H_2O_2 had no significant effect on the content in milk of nicotinic acid, thiamine, riboflavin, vitamin B_6, pantothenic acid, folic acid, vitamin B_{12}, vitamin A or β-carotene, or on that in cheese and whey prepared from the milk. Electrophoretic studies of the proteins from the cheeses showed only minor differences that were not attributed to the treatment with H_2O_2. Rat-growth tests showed that treatment with H_2O_2 under these conditions had no marked effect on the protein efficiency ratio of milk or of cheese or whey prepared from it. The method is, however, less sensitive to small differences than the balance-sheet method used in our experiments (see Henry *et al.* 1947–48). These authors did not comment on the quality of the cheeses made from the H_2O_2-treated milks. However, it has been found that under certain conditions treatment of milk with low concentrations of H_2O_2 can produce cheese of better quality than that made from pasteurized milk (Roundy, 1958; Lück, 1956).

Since, apart from a slight reduction in the nutritive value of milk proteins and

possibly a small loss of vitamin B_{12}, the addition of H_2O_2 at low concentration to milk as a preservative has no adverse effect on its nutritive value, it is evident that such treatment may have a place in the dairy industry. The bacteriological findings of this experiment, and in particular the survival of the coli-aerogenes group, support the opinion that H_2O_2 treatment of milk cannot be relied upon to replace pasteurization or other heat treatment in safeguarding milk for consumption in liquid form. However, since the low concentration of 0·05 % (w/v) H_2O_2 used in these experiments had little effect on the nutritive value of the milk and certainly improved its bacterial quality, there seems no reason why it should not be used in tropical countries to ensure that milk reaches the processing plant in good condition.

We thank Dr J. E. Ford for the measurements of 'available' methionine and 'nutritional value' with *Streptococcus zymogenes*, Miss H. R. Chapman and the Staff of the Experimental Dairy at the N.I.R.D. for their help in handling the milk during the H_2O_2 treatment and Mr W. A. Cuthbert and his colleagues in the Bacteriology Department for their helpful advice.

REFERENCES

ANALYTICAL METHODS COMMITTEE (1959). *Analyst*, **84**, 356.
COUSINS, C. M., HOY, W. A. & CLEGG, L. F. L. (1960). *J. appl. Bact.* **23**, 359.
F.A.O. (1957). *Report on the meeting of experts on the use of hydrogen peroxide and other preservatives in milk.* FAO/57/11/8655. Interlaken, September.
FORD, J. E. (1960). *Brit. J. Nutr.* **14**, 485.
GREGORY, M. E., FORD, J. E. & KON, S. K. (1958). *J. Dairy Res.* **25**, 447.
HENRY, K. M. & KON, S. K. (1956). *Brit. J. Nutr.* **10**, 39.
HENRY, K. M., KON, S. K., LEA, C. H. & WHITE, J. C. D. (1947–48). *J. Dairy Res.* **15**, 292.
HENRY, K. M., KON, S. K. & WATSON, M. B. (1937). *Milk and Nutrition Part I*, p. 37. Reading: National Institute for Research in Dairying.
KON, S. K. & THOMPSON, S. Y. (1957). *Milchwissenschaft*, **12**, 166.
LÜCK, H. (1956). *Dairy Sci. Abstr.* **18**, 363.
LÜCK, H. & SCHILLINGER, A. (1958). *Z. LebensmittUntersuch.* **107**, 512; **108**, 341.
MITCHELL, H. H. (1923–24). *J. biol. Chem.* **58**, 873.
MITCHELL, H. H. & CARMAN, G. G. (1926). *J. biol. Chem.* **68**, 183.
NAMBUDRIPAD, V. K. N., LAXMINARAYANA, H. & IYA, K. K. (1952). *Indian J. Dairy Sci.* **5**, 135.
ROSELL, J. M., DAGÁ, C. & FERNANDEZ, J. (1959). *Proc. XVth Int. Dairy Congr.* **1**, 418.
ROUNDY, Z. D. (1958). *J. Dairy Sci.* **41**, 1460.
SARETT, H. P. & CHELDELIN, V. H. (1944). *J. biol. Chem.* **155**, 153.
TEPLY, L. J., DERSE, P. H. & PRICE, W. V. (1958). *J. Dairy Sci.* **41**, 593.

Printed in Great Britain

The effect of the presence of penicillinase producing micrococci on the growth of cheese and yogurt starters in milk contaminated with penicillin

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(*Received* 27 *February* 1961)

Summary. Coagulase-negative micrococci isolated from the cow's udder, when cultivated together with cheese and yogurt starters in milk containing 0·3 i.u./ml of penicillin, contributed sufficient penicillinase to permit normal rates of acid production by the starters. Subcultures of such 'mixed' cultures also grew at normal rates in milk contaminated with penicillin at the same level.

The possibility of using such micrococci to protect the starter organisms in both the preparation of bulk starters and during manufacture of cheese and yogurt is discussed.

Difficulties in the manufacture of dairy products caused by antibiotics in the milk received from farms continue to increase. Ridgway (1960) found, in a restricted area, that penicillin is still the main antibiotic found in milk. Several ways of dealing with the problem have been proposed: segregation of penicillin contaminated milk at creameries producing cheese and yogurt, and perhaps also at drying plants which could make penicillin free skim-milk powder for use in preparing cheese and yogurt starters; routine treatment of starter milk with penicillinase, but not the manufacturing milk because of the expense; and the use of single-strain penicillin resistant starters. It occurred to us that a penicillinase producing, but innocuous, bacterium could be grown along with the starter strains to inactivate any level of penicillin likely to occur in manufacturing milk.

Since Abraham & Chain (1940) showed the presence in some bacteria of an enzyme, which they named penicillinase, capable of destroying the antibacterial action of penicillin, many species have been found to produce this enzyme. Abraham *et al.* (1941) observed that *Staphylococcus aureus*, made resistant to penicillin *in vitro*, did not contain penicillinase, which was confirmed amply by later workers. Bellamy & Klimek (1948), however, found that some staphylococci with a very high induced penicillin resistance produced penicillinase but only when the medium contained penicillin. Thus as naturally occurring resistant strains were more likely to be penicillinase producers we examined some strains of micrococci isolated from cow's udders by Mr T. M. Higgs of this Institute.

Two strains with high rates of penicillinase production were selected, briefly

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characterized and then used in experiments with cheese and yogurt starters. As the micrococci only grew feebly in milk, the minimum number of micrococci needed to nullify the effect of 0·3 I.U./ml of penicillin in milk was first determined for the two starters.

The actual number of micrococci added to the bulk starters had to be sufficient not only to inactivate 0·3 I.U./ml of penicillin in the starter medium, but also to permit sufficient carry-over of micrococci into the manufacturing milk to inactivate the same level of penicillin. The laboratory findings were tested by making Cheddar cheese on a small scale.

METHODS

Starter strains

A single strain cheese starter organism, *Streptococcus lactis* ML_3 (NCDO 763) was used. It was subcultured daily in litmus milk using a 1 % inoculum and incubation for 24 h at 22 °C. A mixed culture of *Str. thermophilus* and *Lactobacillus bulgaricus*, in use for the commercial production of yogurt, was kindly supplied by Dr R. J. MacWalter, United Dairies, Ltd. It was subcultured daily in litmus milk (2 % inoculum) and incubated for 18 h at 37 °C. Cheese starters are usually grown at 22 °C for 18–24 h, but we used an incubation temperature of 30 °C to shorten the time, thus permitting readings to be taken within a working day.

Media

The media used for identifying and characterizing the micrococci included the following. The pH of all media was 6·8 unless otherwise stated.

Yeast dextrose broth (YDB) and the corresponding solid medium with agar (YDA) contained dextrose, 0·5 %; Evans peptone, 1·0 %; Oxoid Lab Lemco, 1 %; yeast extract (Yeastrel), 0·3 %; NaCl, 0·5 %; agar (Oxoid No. 3), 1·5 %. Nutrient broth (NB) and the corresponding solid medium (NA) contained Evans peptone, 1·0 %; Oxoid Lab Lemco, 1 %; NaCl, 0·5 %; agar (Oxoid No. 3), 1·5 %. The selective medium for micrococci consisted of nutrient agar with 6 % NaCl.

The synthetic medium used for testing the micrococci for ability to utilize different sources of nitrogen consisted of a basic medium to which was added either 0·1 % of NH_4Cl, 0·1 % of urea or 0·1 % of peptone, the pH being adjusted to 7·0. The basic medium contained glucose, 0·4 %; $MgSO_4$, 0·1 %; KH_2PO_4, 0·3 %; Na_2HPO_4, 0·6 %; NaCl, 0·1 %; brom-cresol purple, 0·1 %; agar 1·5 %.

Horse blood agar was used for the determination of haemolysis and consisted of NA and 5 % horse blood. Starch hydrolysis was determined on NA + 0·1 % soluble starch. Peptone water contained Evans peptone, 1 %; salt, 0·5 %; and brom-cresol purple, 0·25 %; sugars, 2 %. Dextrose broth (DLB) was used for the salt tolerance tests and consisted of Evans peptone, 1 %; Oxoid Lab Lemco, 1 %; dextrose, 1 %.

Lipolysis was observed on tributyrin and butterfat agar consisting of Evans peptone, 1 %; Oxoid Lab Lemco, 0·3 %; yeast extract (Yeastrel), 0·3 %; NaCl, 0·5 %; tryptone (Bacto), 1 %; and either 1 % tributyrin or butterfat. The pH was adjusted to 6·0 with lactic acid.

Laboratory techniques

Growth in broth was determined turbidimetrically, using a Biochem absorptiometer with a no. 58 filter. Bacterial counts were made in duplicate on YDA at 30 °C for *Str. lactis* and on NA + NaCl at 37 °C for the micrococci. The method of Miles & Misra (1938) was employed.

Because of the large numbers of milk cultures in duplicate required, it was not practicable to measure acidities by titration. Instead the times of reduction and clotting of litmus milk were determined.

When required, cultures of the micrococci in L-shaped tubes plugged with cotton wool were aerated during growth by shaking in a Microid Flaskshaker.

Penicillin

Tablets of the potassium salt of benzylpenicillin with sodium citrate containing 15000 I.U. of penicillin (Burroughs Wellcome and Co.) were dissolved in distilled water to give the required concentration and stored in the refrigerator for a maximum time of 36 h. For higher concentrations of penicillin, vials containing crystalline sodium salt of benzylpenicillin (Glaxo Ltd.) containing 10^6 I.U. of penicillin were used.

Sentest tablets containing 2·5 I.U. of penicillin (Evans no. 2) were used in the usual way on agar to determine the penicillin sensitivity of the micrococci.

We shall refer to penicillin as P., concentration of penicillin in I.U./ml as I.U.P./ml, and penicillinase as P-ase.

Determination of penicillin sensitivity

Two methods were used for determining the minimum concentration of P. which affected the growth of the cheese and yogurt starters in litmus milk. In the less precise method the minimum concentration of P. which prevented clotting of the milk in 18 or 24 h was noted. Duplicate 10 ml quantities of milk containing increasing concentrations of P. were inoculated either at the rate of 1 % with a clotted culture of strain ML_3 (and then incubated at 30 °C) or 2 % with a clotted culture of the yogurt strains (subsequently incubated at 37 °C).

For the more precise method the concentration of P. which permitted clotting of the milk at the same time as control cultures without P. was recorded as the P. sensitivity of the starter.

The sensitivity of the micrococci to P. was determined in YDB using a standard inoculum of 10^6 cells/ml. Growth in YDB containing P. and in P.-free medium was compared by taking hourly readings with the Biochem absorptiometer.

Comparative penicillinase activity of different strains and cultures of micrococci

Using ML_3 as a test organism the P-ase activity of equal numbers of micrococci from different cultures was compared. Increasing concentrations of P. were added to litmus milk, and after adding a standard inoculum of 10^6 micrococcal cells/ml the tubes and contents were held at 30 °C for 2 h. The tubes were then inoculated at the

rate of 1 % with a clotted culture of strain ML_3. For each culture of micrococci the maximum concentration of P. originally present, which was destroyed to the level which permitted clotting of the litmus milk by strain ML_3 within 18 or 24 h, was regarded as a measure of the P-ase activity of the micrococcus preparation.

The determination of the concentration of micrococci required to inactivate 0·3 I.U./*ml of penicillin in milk*

A fixed level of 0·3 I.U.P./ml was chosen as being above what is likely to be found in practice. Decreasing numbers of micrococci/ml were added to litmus milk plus 1 % of a clotted culture of strain ML_3 or 2 % of the yogurt culture and then held at 30 or 37 °C respectively. The minimum concentration of micrococci required to inactivate the penicillin, without resorting to a contact period, to the extent that the starter could reduce and clot the litmus milk in the same time as in P.-free milk was recorded.

The mixed cultures at the end of these tests contained varying numbers of micrococci. Subcultures inoculated at the rates appropriate to the starter organisms were made in fresh tubes of litmus milk containing 0·3 I.U.P./ml, and incubated at the appropriate temperature. The times of reduction and clotting of these cultures were compared with those of P.-free control cultures.

The two stages of these experiments represented the preparation of bulk starters and the manufacture of cheese and yogurt.

EXPERIMENTAL

Penicillinase producing micrococci

Thirty strains of micrococci were tested for P-ase production. They were Gram positive, catalase positive, coagulase negative, grew in the presence of 6 % NaCl and formed white or yellow pigmented colonies on agar. The isolates were streaked on NA plates radially towards a cup containing about 100 I.U. of penicillin. Strains which grew right up to the source of P. were restreaked on NA containing 6 % NaCl and picked into NB. Altogether nine P-ase producing strains were isolated in this way. It was found that the cell free medium contained very little activity against P.; the bulk of the P-ase is, therefore, intracellular. The destruction of the activity against P. at the same temperatures at which the micrococci were killed supports this.

On the basis of their biochemical reactions the nine strains fell into two distinct groups. The strain from each group producing the most P-ase was selected for the experiments with starters.

Fig. 1 gives the growth curves of these two strains, 34/3 and 24/1H/4, in YDB and in YDB containing 0·5 I.U.P./ml. There was some delay of growth in the presence of P. but subsequently the end growth determined by turbidity and cell count was the same.

Table 1 gives the results of the biochemical tests made with strains 34/3 and 24/1H/4. The main differences were in growth in milk, proteolysis of milk, lipolysis, utilization of urea as sole source of nitrogen, haemolysis, hydrolysis of urea and reduction of nitrate. From the sugar reactions it may be seen that 24/1H/4 fermented lactose in peptone water, but not in milk (no acid formed). Strain 24/1H/4

only grew in milk when a source of degraded protein (peptone, casein digest) was added.

The system of Hucker (1924) is still followed in *Bergey's Manual* (Breed, Murray & Smith, 1957), but attempts at classification have been made since by Abd-el-Malek & Gibson (1948) and Shaw, Stitt & Cowan (1951). Identification of the micrococci appears to be still far from satisfactory and for our purposes a distinction by proteolysis and lipolysis may be more useful in view of the nature of the dairy products for which starters are used.

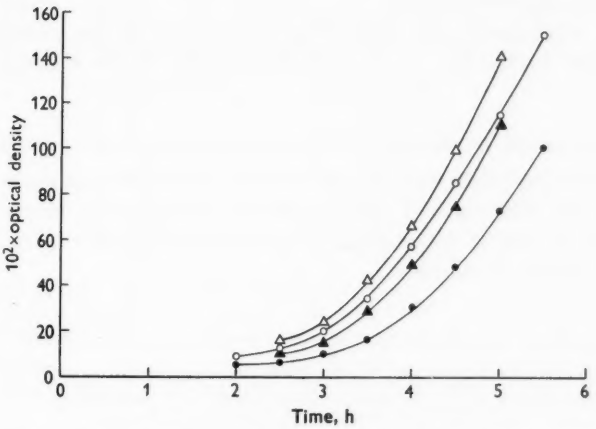

Fig. 1. Penicillin resistance of two strains of micrococci in YDB. Strain 34/3: ●—●, 0·5 I.U./ml of penicillin; ○—○, no penicillin. Strain 24/1H/4: ▲—▲, 0·5 I.U./ml of penicillin; △—△, no penicillin.

Table 1. *Characterization of micrococci, strains* 24/1H/4 *and* 34/3

Colony pigmentation	24/1H/4 Yellow	34/3 White
Haemolysis on horse blood agar	α	γ
Urea hydrolysis	−	+
Nitrate reduction	−	+
Growth in DLB + 10% NaCl	+	+
Growth in DLB + 12% NaCl	+	+
Gelatin liquefaction	+	+
Litmus milk	No change	Reduced clotted peptonized
Sole source of nitrogen		
Ammonia	+	+
Urea	−	+
Lipolysis		
On tributyrin agar	+	+
On butterfat agar	−	+
Nile blue sulphate reaction on butterfat agar	−	+
Mannitol	+	−
Glycerol	−	+

Both strains were Gram +, catalase +, coagulase −, did not hydrolyse starch, and in peptone water fermented lactose, maltose, and sucrose.

The penicillin sensitivity of Streptococcus lactis ML_3 *and the yogurt starter*

Preliminary experiments showed the sensitivity of strain ML_3 to be 0·01 I.U.P./ml and of the yogurt 0·005 I.U.P./ml. At those concentrations the penicillin contaminated milk was clotted at the same time as the control which with a 1% inoculum of strain ML_3 was 7–8 h at 30° and with a 2% inoculum of the yogurt was 4–5 h at 37 °C. Further readings at 18 h established that strain ML_3 clotted the milk in the presence of 0·17 I.U.P./ml. Propagation of such cultures in P.-free milk showed normal rates of acid development.

From these experiments it is clear that the penicillin in the manufacturing milk must be reduced to the lower values if the activity of the starters is not to be impaired.

Penicillinase production by micrococci, strains 24/1H/4 *and* 34/3

Duthie (1944) found that the P-ase production of his organisms was increased 20- to 30-fold by the presence of P. in the growth medium, the enzyme P-ase being adaptive. Pollock (1953) demonstrated that aeration further increases the P-ase production of aerobic organisms.

Table 2. *The effect of the presence of a constant inoculum of micrococcus* 24/1H/4 *grown with aeration in YDB containing various amounts of penicillin, on the clotting in* 18 h *by* Streptococcus lactis ML_3 *of litmus milk containing increasing levels of penicillin*

Level of penicillin in litmus milk inoculated with micrococci and *Str. lactis* ML_3, I.U./ml	Appearance of the litmus milk after 18 h, when micrococci were grown in YDB containing 0–25 I.U./ml of penicillin			
	0	0·5	5	25
0	X	X	X	X
100	X	X	X	X
150	R	X	X	X
200	NR	X	X	X
300	—	R	X	X
400	—	NR	R	X
600	—	—	NR	R
800	—	—	—	NR

X = clotted; R = reduced only; NR = not reduced.
Cell count of inoculated medium/ml: strain ML_3, $2{\cdot}5 \times 10^7$; strain 24/1H/4, 1×10^6.

Table 2 shows the effect of an increasing amount of P. in the medium (YDB) on the P-ase production of strain 24/1H/4. When grown without penicillin in the medium, 10^6 cells/ml reduced between 100 and 150 I.U.P./ml sufficiently to permit strain ML_3 to grow. In the presence of 25 I.U.P./ml in the medium 400–500 I.U.P./ml were reduced. It is clear therefore that the presence of increasing amounts of P. in the medium induced higher production of P-ase.

Similar experiments, but without aeration, showed an appreciable reduction of P-ase production thus confirming the findings with other bacterial species.

During this work it was found that the age of the culture had a considerable effect on the power of the same number of cells to inactivate P. This is illustrated in Table 3.

In this experiment maximum P-ase production was achieved after 7 h which represents the end of the logarithmic phase of growth.

Similar results to the above were obtained with strain 34/3.

Table 3. *The effect of the presence of a constant inoculum of micrococcus* 24/1H/4 *from cultures incubated for various times with aeration in YDB* + 2 I.U./*ml of penicillin, on the clotting in* 24 *h by* Streptococcus lactis *ML_3 of litmus milk containing increasing levels of penicillin*

Level of penicillin in litmus milk inoculated with micrococci and *Str. lactis*, ML_3 I.U./ml	Appearance of the litmus milk after 24 h, when the micrococcal cultures were incubated for		
	5 h	7 h	10 h
0	X	X	X
200	X	X	X
300	NR	X	R
400	—	X	NR
600	—	R	—
800	—	NR	—

X = clotted; R = reduced only; NR = not reduced.
Cell count of inoculated medium/ml: strain ML_3, 2×10^7; strain 24/1H/4, 1×10^6.

Penicillin inactivation in subcultures of mixed cultures of micrococci and Streptococcus lactis *ML_3*

The experiments described so far show that P-ase producing micrococci can protect strain ML_3 against high levels of penicillin when the micrococci are added sometime before the inoculum of streptococci. High numbers of the micrococci up to 10^9 cells/ml can easily be obtained in aerated cultures. It was found experimentally that 10^3 micrococci/ml were sufficient to protect against 0·3 I.U.P./ml of milk. While such relatively low concentrations of micrococci would protect the bulk starter, a higher number of cells could be used to protect the cheesemilk against penicillin contamination by carrying over sufficient micrococci with the starter inoculum (1–2 %) into the cheesemilk. The choice in practice would depend very largely on having available strains of micrococci which had been shown to have no harmful effect on the cheese.

The following experiments illustrate some of the conditions necessary for the micrococci to protect *Str. lactis* ML_3 against 0·3 I.U.P./ml in both the milk of the original culture and the first subculture.

Strain 24/1H/4 was grown with aeration in the presence of 25 I.U.P./ml and standardized by optical density to allow known diminishing numbers to be added to tubes of milk containing 0·3 I.U.P./ml. A 1 % inoculum of a culture of strain ML_3 was added and the tubes observed for clotting at 30 °C (Table 4). These mixed cultures were subcultured after 7 and 18 h into tubes of milk again containing 0·3 I.U.P./ml and incubated at 30 °C (Table 5).

From Tables 4 and 5 it is clear that the micrococcus grew slowly for some time after inoculation but tended to die out on prolonged incubation, probably due to poor acid tolerance. An initial inoculum of 10^5 to 10^6 cells/ml was sufficient to protect the subculture against 0·3 I.U.P./ml.

Somewhat smaller concentrations of strain 34/3 were required because it grew better in milk.

Table 4. *Protection of* Streptococcus lactis *ML_3 by decreasing number of cells of micrococcus* 24/1H/4 *in litmus milk containing* 0·3 I.U./*ml of penicillin (equivalent to bulk starter)*

	Appearance of litmus milk at 3 h	4½ h	7 h	Count/ml of 24/1H/4 after 7 h	18 h
	No micrococci				
Strain ML_3 $2·5 \times 10^7$ cells/ml					
No penicillin in the litmus milk	NR	R	X	—	—
0·3 I.U. of penicillin/ml of litmus milk	NR	NR	NR*	—	—
	Plus micrococci				
0·3 I.U. of penicillin and $2·5 \times 10^7$ cells of strain ML_3/ml of litmus milk + 24/1H/4 cells/ml					
$2·5 \times 10^7$	R	R	X	$1·2 \times 10^8$ (1)	5×10^7 (1*a*)
1×10^7	R	R	X	$7·5 \times 10^7$ (2)	$3·5 \times 10^7$ (2*a*)
5×10^6	R	R	X	4×10^7 (3)	2×10^7 (3*a*)
$2·5 \times 10^6$	NR	R	X	2×10^7 (4)	$1·2 \times 10^7$ (4*a*)
1×10^6	—	R	X	8×10^6 (5)	$1·5 \times 10^6$ (5*a*)
5×10^5	—	R	X	3×10^6 (6)	$1·3 \times 10^6$ (6*a*)

* Not clotted at 18 h.

X = clotted; R = reduced only; NR = not reduced.

After 7 and 18 h the cultures containing micrococci were subcultured (1 % inoculum) into fresh tubes of litmus milk containing 0·3 I.U./ml of penicillin. The numbers in brackets relate the cultures to the results in Table 5.

Penicillin inactivation in subcultures of mixed cultures of micrococci and the yogurt starter

Similar experiments to those just described for *Str. lactis* ML_3 were done with the yogurt starter, except that a 2 % inoculum was used and the incubation temperature was 37 °C. The results for the first propagation of the micrococci and the starter, are summarized in Table 6. The results for the first subculture of the mixed cultures are summarized in Table 7.

From Table 6 it may be seen that a relatively high number of micrococci ($2·5 \times 10^7$/ml) was required to inactivate 0·3 I.U.P./ml to allow clotting in the same time as the control containing no P. From Table 7, it seems that lower concentrations of micrococci were effective in the subcultures. (If, however, a delay of about 1 h in the manufacturing time is acceptable a lower concentration of micrococci would be sufficient.) Furthermore, ageing of the bulk starter reduced the count of micrococci in the final culture to below 10/ml.

Similar results were obtained with strain 34/3, but because of its proteolytic and lipolytic properties this strain may be less acceptable than strain 24/1H/4.

These results are less consistent than those obtained with *Str. lactis* ML_3, and this may be related to the behaviour of the separate species in the yogurt starter and also to the higher acidity of yogurt. No attempt was made to determine the proportion of *Str. thermophilus* and *Str. bulgaricus* in the cultures propagated in the presence of P.

Table 5. *Propagation of mixed cultures (MS, see Table 4 for sources of the numbered cultures) of* Streptococcus lactis ML_3 *and micrococcus* 24/1H/4 *in milk containing* 0·3 I.U./*ml of penicillin (equivalent to cheese manufacture)*

(*a*) Propagation of MS-culture, 7 h old

	Appearance of litmus milk at		Count/ml of 24/1H/4 after 7 h
	4 h	7 h	
Control. 2×10^7 cells of ML_3/ml, no penicillin in the litmus milk	R	X	—
1% inoculum of MS-culture into litmus milk (containing 0·3 I.U./ml of penicillin) giving approx. 10^7 ML_3 cells/ml + 24/1H/4 cells/ml			
(1) $1{\cdot}2 \times 10^6$	R	X	2×10^6
(2) $7{\cdot}5 \times 10^5$	R	X	$1{\cdot}2 \times 10^6$
(3) 4×10^5	R	X	7×10^5
(4) 2×10^5	R	X	4×10^5
(5) 8×10^4	R	X	$1{\cdot}5 \times 10^5$
(6) 3×10^4	R	X	7×10^4

(*b*) Propagation of MS-culture, 18 h old

	Appearance of litmus milk at				Count/ml of 24/1H/4 after 8½ h
	5 h	7 h	8 h	8½ h	
Control. 2×10^7 cells of ML_3/ml, no penicillin in the litmus milk	R	R	X	X	—
1% inoculum of MS-culture into litmus milk (containing 0·3 I.U./ml of penicillin) giving approx. 10^7 ML_3 cells/ml + 24/1H/4 cells/ml					
(1*a*) 5×10^5	R	R	X	X	$1{\cdot}2 \times 10^6$
(2*a*) $3{\cdot}5 \times 10^5$	R	R	X	X	4×10^5
(3*a*) 2×10^5	R	R	X	X	3×10^5
(4*a*) $1{\cdot}25 \times 10^5$	R	R	X	X	2×10^5
(5*a*) $1{\cdot}5 \times 10^4$	R	R	X	X	3×10^4
(6*a*) $1{\cdot}3 \times 10^4$	NR	R	R	X	1×10^4

X = clotted; R = reduced only; NR = not reduced.

CHEESE EXPERIMENT

At the time of this small-scale Cheddar cheese experiment (40 gal of milk in each vat) strain 34/3 was the most active strain of micrococcus available. It was grown in YDB with aeration in the presence of 25 I.U.P./ml and inoculated into bulk starter milk to give 3×10^7 cells/ml. The milk contained 0·3 I.U.P./ml. After inoculation with strain ML_3 (1%) the mixed culture was grown for 18 h at 30°C. At this stage the mixed starter contained 2×10^7 micrococcal cells/ml, and as 1¼% of starter was used the cheesemilk contained approximately 10^5 micrococci/ml.

Three vats were made: A—control, ML_3 starter only, cheesemilk without penicillin; B—control, ML_3 starter only, cheesemilk containing 0·15 I.U.P./ml; C—mixed starter prepared in milk containing 0·3 I.U.P./ml, the cheesemilk containing 0·15 I.U.P./ml.

Table 6. *Protection of a commercial yogurt culture by decreasing number of cells of micrococcus 24/1H/4 in litmus milk containing 0·3 I.U./ml of penicillin (equivalent to bulk starter)*

	Appearance of litmus milk at			Count/ml of 24/1H/4 after	
	4½ h	5 h	5½ h	5½ h	18 h
No micrococci					
2% inoculum of yogurt					
No penicillin in the litmus milk	X	X	X	—	—
0·3 I.U. of penicillin/ml of litmus milk	NR	NR	NR	—	—
Plus micrococci					
2% inoculum of yogurt, 0·3 I.U. of penicillin/ml of litmus milk + 24/1H/4 cells/ml					
$2{\cdot}5 \times 10^7$	X	X	X	$2{\cdot}5 \times 10^8$ (1)	2×10^4 (1*a*)
1×10^7	NR	X	X	$1{\cdot}25 \times 10^8$ (2)	$1{\cdot}5 \times 10^4$ (2*a*)
5×10^6	—	X	X	1×10^8 (3)	$6{\cdot}5 \times 10^3$ (3*a*)
$2{\cdot}5 \times 10^6$	—	NR	X	5×10^7 (4)	4×10^3 (4*a*)
1×10^6	—	—	X	$2{\cdot}5 \times 10^7$ (5)	3×10^3 (5*a*)
5×10^5	—	—	X	$1{\cdot}5 \times 10^7$ (6)	< 10 (6*a*)

X = clotted; R = reduced only; NR = not reduced.

After 5½ h and 18 h the cultures containing micrococci were subcultured (2% inoculum) into fresh tubes of litmus milk containing 0·3 I.U./ml of penicillin. The numbers in brackets relate the cultures to the results in Table 7.

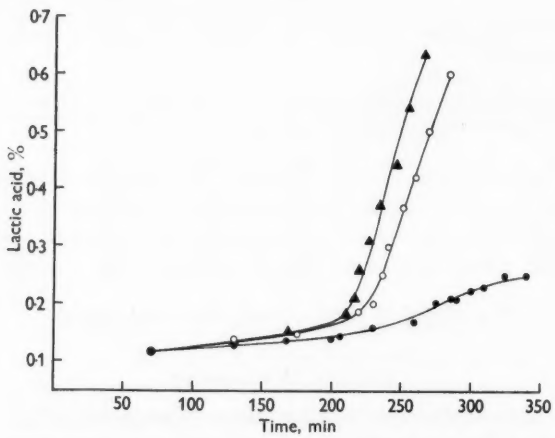

Fig. 2. Cheese experiment. ○—○, Control, strain ML_3 only; ●—●, Negative control, strain ML_3 only, milk containing 0·15 I.U./ml of penicillin. ▲—▲, Experimental vat, mixed starter of strain ML_3 and of micrococci, strain 34/3, milk containing 0·15 I.U./ml of penicillin.

Vat A, the control, gave normal acid development and vat C was slightly faster, conceivably due to the proteolytic properties of micrococcus 34/3. The negative control vat B, containing 0·15 I.U.P./ml, failed completely (Fig. 2). The mixed culture proved effective therefore against the level of contamination of penicillin which powerfully affected starter ML_3. We chose 0·15 I.U.P./ml instead of 0·3 as in the laboratory experiments, because we intended only to slow down acid development.

Table 7. *Propagation of mixed cultures (MY, see Table 6 for sources of the numbered cultures) of the yogurt and micrococcus 24/1H/4 in milk containing 0·3 I.U./ml. of penicillin (equivalent to yogurt manufacture)*

(*a*) Propagation of MY-culture, 5½ h old

	Appearance of litmus milk at		Count/ml of 24/1H/4 at
	4 h	5 h	5½ h
Control. 2% inoculum of yogurt, no penicillin in the litmus milk	X	X	—
2% inoculum of MY-culture into litmus milk (containing 0·3 I.U./ml of penicillin) +24/1H/4 cells/ml			
(1) 5×10^6	X	X	$1{\cdot}2 \times 10^7$
(2) $2{\cdot}5 \times 10^6$	X	X	1×10^7
(3) 2×10^6	X	X	3×10^6
(4) 1×10^6	NR	X	2×10^6
(5) 5×10^5	—	X	8×10^5
(6) 3×10^5	—	X	7×10^5

(*b*) Propagation of MY-culture, 18 h old

	Appearance of litmus milk at				Count/ml of 24/1H/4 at
	5 h	6½ h	7 h	7½ h	7½ h
Control. 2% inoculum of yogurt, no penicillin in the litmus milk	X	X	X	X	—
2% inoculum of MY-culture into litmus milk (containing 0·3 I.U./ml of penicillin) +24/1H/4 cells/ml					
(1*a*) 4×10^2	NR	X	X	X	1×10^3
(2*a*) 3×10^2	—	X	X	X	6×10^3
(3*a*) $1{\cdot}3 \times 10^3$	—	X	X	X	< 10
(4*a*) 80	—	NR	X	X	< 10
(5*a*) 60	—	—	X	X	< 10
(6*a*) < 0·2	—	—	NR	X	< 10

X = clotted; R = reduced only; NR = not reduced.

The cheeses from vats A and C were judged at 8 weeks but scored low, C being, however, judged slightly inferior to A. The cheese from vat C had a count of 10^3 micrococci/g on nutrient salt agar at 8 weeks, and the cheese from vat A a count of < 10/g.

DISCUSSION

From the results presented it appears that the addition of P-ase producing micrococci, providing they can be shown to be innocuous, to milk contaminated with P. would be of practical value. The strains of micrococci used in the present experiments were isolated from the cow's udder, but a more likely source of suitable strains would perhaps be cheese of good quality. Recently we have tested twenty-nine strains of micrococci isolated from commercial Cheddar cheese by Dr J. G. Franklin and Dr M. E. Sharpe of this Institute. Three strains produced P-ase, but as with the strains described in this paper little is known of their influence on the quality of cheese when present in the starters in high numbers.

From the results obtained in the laboratory it appears that 10^3 micrococci/ml could protect a bulk starter against as much as 0·3 I.U.P./ml in the starter milk. As there is no difficulty in obtaining a culture of 10^9 cells/ml, about 0·5 ml could protect 100 gal of starter milk. Contamination of the cheesemilk at the same level would require 10^5 to 10^6 micrococci/ml equivalent to 50–500 ml of a 10^9/ml micrococcal culture as inoculum for 100 gal of starter.

For the protection of a yogurt culture, however, a relatively high concentration of micrococci (10^7 cells/ml) is required to avoid delay in clotting. Much lower concentrations would suffice if a slight delay in manufacture is tolerated and it appears feasible that an inoculum of 50–100 ml in the starter milk would be sufficient to protect 100 gal of yogurt.

Both strains of micrococci 24/1H/4 and 34/3 have been deposited with the National Collection of Dairy Organisms.

The authors wish to thank Mr W. A. Cuthbert, M.Sc., for his stimulation and his interest in this work; and Miss H. R. Chapman for making the cheese.

REFERENCES

ABD-EL-MALEK, Y. & GIBSON, F. (1948). *J. Dairy Res.* **15**, 249.
ABRAHAM, E. P. & CHAIN, E. (1940). *Nature, Lond.*, **158**, 818.
ABRAHAM, E. P., FLETSCHER, C. M., FLOREY, H. W., GARDENER, A. D., HEATLEY, N. G. & JENNINGS, M. A. (1941). *Lancet*, ii, 177.
BELLAMY, W. D. & KLIMECK, J. W. (1948). *J. Bact.* **55**, 153.
BREED, R. S., MURRAY, E. G. D. & SMITH, N. R. (1957). *Bergey's Manual of Determinative Bacteriology.* London: Baillière, Tindall and Cox.
DUTHIE, E. S. (1944). *Brit. J. exp. Path.* **25**, 96.
HUCKER, G. J. (1924). *N.Y. State Agric. Expt. Sta. Tech. Bull.* no. 101.
MILES, A. A. M. & MISRA, S. S. M. (1938). *J. Hyg., Camb.*, **38**, 732.
POLLOCK, A. V. (1953). *Brit. J. exp. Path.* **34**, 251.
RIDGWAY, J. D. (1960). *J. Soc. Dairy Tech.* **13**, 197.
SHAW, C., STITT, J. M. & COWAN, S. T. (1951). *J. gen. Microbiol.* **5**, 1010.

Printed in Great Britain

Reviews of the progress of dairy science

Section A. Physiology

Genetics of Dairy Cattle

(*Received* 7 *February* 1961)

This is not intended to be in any way a complete review of all papers on dairy cattle genetics in the last few years but rather a concentration on the topics that have excited the most interest in that time and on the papers that have been the most valuable and informative. As such, it is bound to be coloured by my own interests and activities.

BLOOD GROUPS AND PROTEINS

Perhaps the most interesting new development in recent years has been the use of immunological and biochemical techniques to enable us to detect individual genes segregating in our cattle breeds. The start was made in cattle blood groups and the pioneer work by Irwin, Stormont & Owen was done almost 20 years ago (1, 2). Probably the best recent review is by Stormont (3). We know now ten different loci in cattle, controlling the reaction of the red cells to some fifty or so different reagents. The different loci vary considerably in their complexity. At five of them, we know of only two alternative alleles, and at a further three we know of the segregation of up to four alleles. But at two of the loci, the so called B and C systems, the variety is tremendous. In the relatively simpler C system, seven different reagents are generally used but at least twenty-four different alleles, each controlling a different reaction pattern, are known to exist. The B system shows the greatest complexity. It governs the reactions to about twenty different reagent antisera. Over 200 different alleles are known at this locus and every time a new breed of cattle is looked at, some new alleles are found.

Very recently new biochemical techniques have appeared which allow us to distinguish very small differences between protein molecules. These techniques are extensions of the well-known techniques of paper chromatography and depend on the migration of the protein molecules under an electrical gradient in either specially treated papers or blocks of starch gel. The separation depends in essence on the existence of different electrical charges on the different molecules though some aspects of the physical chemistry are not well understood. In human haemoglobin, one of the first proteins to be so analysed, the variant types have been carefully investigated and it appears probable that, at the level of molecular structure, we are dealing in the two most frequent forms merely with the substitution of one amino acid for another at one place in one of the two pairs of protein chains which constitute this molecule.

In cattle breeds, two alleles are known controlling haemoglobin type in temperate breeds of cattle though another has recently been found in tropical cattle (4,5). In blood serum proteins, genetic variation has been found in the so-called 'transferrins',

whose function is to remove iron ions from the blood, by Smithies & Hickman in Canada (6) and by Ashton in Great Britain (7). Here we know only one locus with three alleles segregating in temperate breeds and another two in Indian zebus.

In milk proteins, genetic variation is known in two specific components. Two alleles have been found in temperate breeds governing the kind of β-lactoglobulin in milk (8). Being an easily crystallizable protein, this polymorphism has been investigated biochemically rather more than the others. The physical differences between the two kinds of β-lactoglobulin are rather small and it seems possible that the situation may be closely parallel to that in human haemoglobin. Lastly, variation is known in α-lactalbumin but only in tropical breeds of cattle.

At all these loci, the rule governing the inheritance is that, if an allele producing a certain protein or blood-group substance is carried by an animal, the relevant protein or reaction will always be detected. Hence parentage testing depends primarily on looking for reactions or proteins in the offspring which are not detected in either parent.

It might be mentioned that similar variation is known in other species of domestic animals such as pigs, sheep and poultry. In sheep breeds, there is an interesting segregation of a recessive gene affecting the potassium level in red cells.

Apart from immunological and biochemical interest, the various biochemical polymorphisms are of importance for three reasons. First, we have the direct practical use in detecting parentage errors. The possibility of detection depends on the genetic variability actually present in a breed but, using blood group and transferrins, Rendel (9) and Gahne (10) have shown that in SRB cattle the chance of detecting false parentage is over 80 %. The objective distinction between monozygotic and dizygotic twins unfortunately cannot be done with blood groups because of the effects of anastomosis but the transferrin locus is useful here because the serum proteins are not affected.

The differences between breeds at those loci at which there is much genetic diversity are of great value in adding to existing information about the history of dairy cattle (3,11,12,13,14,15). At the loci at which there are few alleles, the general rule is that breeds differ in the frequency of alleles rather than being fixed for one or the other. The few cases in which this is not so are in fact of great interest in presenting clear-cut differences between breeds. Perhaps the most interesting is the Z′ locus at which only two alleles are known. The reagent was produced by the transfusion of a cow of a European breed with blood from a Sindhi bull and the Z′ allele is found at a reasonable frequency in all tropical breeds so far examined (which is however not many). In British breeds, it is found at low frequency in Devons, S. Devons, Jerseys and Guernseys but not at all in the others. In Europe, it appears in breeds whose immediate origins lie south of a line drawn from Calais to the Black Sea.

The allele producing haemoglobin of type B (which has no connection with the B locus of blood groups) has a rather similar distribution (16,17,18). Of British breeds, it is found in Jerseys, Guernseys, and S. Devons. It is found in most of the French breeds (but not in the Normandy) and in many tropical breeds. It appears then to have been introduced to Europe by the same cattle migration which produced the Z′ gene.

The picture is however not all that simple. In transferrins, we know of three alleles

in British cattle, the E gene being rather rare in most breeds (7). It has a frequency of 10% in Ayrshires and of 30% in Swedish Red and White cattle (10), which have a high proportion of Ayrshire blood. The difficulty lies in the fact that it is not known in Jerseys and yet has a high frequency in many tropical breeds.

At the somewhat more complex SU blood group locus, at which six alleles are known, the allele U_2 is interesting. It is at its highest frequency in Dairy Shorthorns with a frequency of about 0·30 and acts as a sort of indicator of Shorthorn blood in the breeds into which it was introduced in the second half of the last century.

Finally, we come to the complex B and C blood-group systems. The C system has not yet been particularly informative in that allele frequencies are difficult to estimate because of the similar reaction patterns controlled by many of them. At the B locus, on the other hand, the twenty or so different reagents give a theoretical possibility of over a million different allelic reaction patterns so that the 200 or so that have been found are often very different in their pattern. More than this, the alleles carried at a reasonable frequency in one breed may not be known at all in another. Any one practised in this can look at the blood type of a cow and have a good chance of guessing what breed it is. In Jerseys, for instance, the allele producing the pattern

$$BGKOY_1A_1E'_3K'$$

has a frequency of about 24% and is not known at all in Herefords in which the allele $Y_1D'I'$ has a frequency of 18%. Here again, the Shorthorn breed has two alleles at a frequency of about 40%, that controlling the reactions $BOY_1A'E'_3$ and that producing no reactions at all (−). One may speculate that at the B locus the genotype of Favourite, who contributed about 50% of the genes in the breed, was $BOY_1A'E'_3/-$.

The blood-group loci not only enable us to infer the past history of our breeds but allow us to see gene frequencies changing in the present. This has been particularly striking in the Red Danish and Dutch Friesian cattle (11,13) in which there has been a great concentration of the blood of a very few ancestors in the last few generations.

There remains the question of the effect of these 'marker' genes on characters of economic importance. This has two aspects. The first most direct one is whether the genes are likely to be useful in a selection programme if animals carrying one particular gene can be shown to be superior to other animals. The 'marker' genes could then be used in selection when other information is lacking, e.g. in deciding which of a group of young bulls to progeny test. The second question is rather more far-reaching. Why do we find this genetic variation still remaining within our breeds? If it is being actively maintained, this would most probably be by selection of heterozygotes. It would therefore be most interesting to know the effect of heterozygosis at these loci—particularly on characters closely related to reproductive ability such as fertility or longevity.

The investigation of the direct effect of blood-group loci is complicated by the multiplicity of possible effects that have to be looked for. This means that the usual rule of twice the standard error for significance at the 5% level is no longer true and that much higher values have to be used in order to be sure that, in the whole data, there is a 5% level covering the extreme of all the comparisons. If this is not taken account of, each set of workers will tend to find a new set of 'significant' effects

which are merely the extremes of a large group of non-significant comparisons. If this is taken account of by increasing the deviation required for significance, it implies that, for an effect of a given magnitude to be accepted as significant, the necessary data may have to be more extensive than for a single comparison.

With these reservations in mind, we can turn to the results of recent investigations. In the East-Friesian breed, it was found that heifers possessing the M gene had a milk yield 332 ± 98 kg less than did other daughters of the same sire (19). This is just significant at the 5 % level, when we bear in mind the multiplicity of the tests. More recent work on the same breed has not been strongly confirmatory of these results.

Working with Friesian cattle in Holland, Bouw (11) found that bulls carrying the Z gene had a progeny test for fat $0{\cdot}059 \pm 0{\cdot}018$ % higher than bulls without Z and other workers have found a slight effect of Z, but not as large as this. The most striking effect so far detected was by Rendel (20) in Swedish Red Cattle where he found that cows with the B allele BOY_2D' had a fat content in their milk $0{\cdot}19 \pm 0{\cdot}03$ % higher than for heifers without this allele and this he confirmed by an analysis of the progeny tests of bulls for fat content. In an analysis of some 2500 lactations from the Danish testing stations, Neimann-Sørensen & Robertson (21) found only one effect with statistical significance. This was the effect of the B allele BOY_1D' (similar but not the same as Rendel's allele) in Red Danish cattle where it increased the fat content by $0{\cdot}064 \pm 0{\cdot}013$ %.

We have not a great deal of evidence as yet on the effects of the loci controlling the other polymorphisms mentioned earlier. Bangham has found no suggestions of associations of haemoglobin genes with production characteristics but Ashton (22) has shown that bulls used at British A.I. stations with the genotype DD at the transferrin locus have a progeny test for yield higher by 26 ± 9 gallons than do bulls of the AA genotype. An interesting effect of the genes affecting β-lactoglobulin is that not only do they control the type of protein produced but that the amount of β-lactoglobulin in the milk depends on the genotype for type of protein.

So far there has been no very convincing evidence to show that heterozygotes at the blood-group loci are markedly superior to homozygotes though, because of the nature of the genes concerned, the analysis can really only be done at three loci. At the transferrin locus, there has been evidence that the segregation ratios are abnormal (though more recent evidence does not confirm this) and that heterozygotes have a higher conception rate.

The factor critical in any judgement of the practical value of 'marker' genes in a selection programme is the proportion of the genetic variance in the character they can be shown to control. In the extensive material on the Red Danish breed, we were able to estimate that only $1{\cdot}5$ % of the observed variation in fat content was controlled by the blood-group loci (in spite of one B allele being found to have a statistically significant effect) and gave a 5 % upper limit of $3{\cdot}7$ % for this (21). This would suggest that not more than 8 % of the genetic variance in fat content is controlled by the blood-group loci, which is not enough to be of practical importance. In Rendel's case in S.R.B. cattle (20), the B locus would then control about 8 % of the total variance and 16 % of the genetic variance, which would approach an important magnitude. In Ashton's case (22), the transferrin locus would control only about 4 %

of the variation between sires in their progeny tests, the latter being mainly genetic in origin. Thus only in Rendel's case does the magnitude of the effect approach the level needed for it to be of practical importance.

ABNORMALITIES

It is not my intention here to attempt to summarize recent analyses of genetic abnormalities in cattle. There are three papers that should be mentioned because they deal with abnormalities from the point of view of the breed as a whole. In Holland, de Groot and his co-workers (23) have produced an excellent monograph on abnormalities that have been found to occur in dairy breeds in that country, giving for each a brief description of the abnormality and of the evidence for its genetic control, both in the literature and from their own evidence in Holland. The other two papers, both rather older, deal with the spread of a recessive abnormality through a breed. Nielsen (24) described the occurrence of a recessive gene causing paralysis in the hind quarters in Red Danish cattle. He estimated that, of bulls born and entered in the herdbook in the mid-forties, about 14 % were heterozygotes for the gene. He was able to trace back the spread of the gene to one particular heterozygous sire used extensively in the 1930's, which in other work has been shown to contribute about 18 % of the genes carried by bulls being used in A.I. around 1950 and therefore comparable with the bulls studied by Nielsen.

The other study, by Donald and his co-workers (25), dealt with the recessive gene causing dropsy in Ayrshire calves in Great Britain. They too were able to trace the spread of the gene, which in 1949 had a frequency of about 5 % in the breed, to the extensive use of sons of one bull born about 1930.

More recently, the emphasis has shifted somewhat from the occurrence of particular abnormalities within breeds to the general situation within the breed, covering all abnormalities. Here there are problems in the collection of the data, as the samples need to be large, and it is often difficult to ensure equivalent coverage in all parts of the sample and adequate estimation of the number of animals 'at risk' in the investigation. It is to be hoped that several investigations now under way covering all births within a certain sample of herds will provide valuable information in this respect.

THE INHERITANCE OF PRODUCTION CHARACTERISTICS

Identical twins have been used for more than 20 years in dairy cattle experiments and have proved a valuable tool in the hands of animal husbandry workers. To the geneticist, they have mainly been the cause of much puzzlement.

Since the early days of twin experimentation it has been obvious that identical twins are extremely similar to one another in milk production, and a superficial conclusion from these results is that variation in milk yield is in the main due to breeding. On the other hand, we have the clear evidence from the analysis of the commercial production records of less close relatives that the heritability was only in the region of 20–30 % compared to around 80–90 % from identical twin studies. This discrepancy has been the cause of much discussion in the last 10 years and one series of experiments has had as its prime object the elucidation of this point.

It seemed possible at one time that there might be an extremely important point here that had been overlooked in previous work, but as more results come in this appears less likely to be so and the difference may be the summation of several small effects. In this connexion, it must be remembered that any heritability is the ratio of a genetic variance to the total variance. The following factors have then to be taken into account. (*a*) Twins are kept in experimental herds in which the level of culling is low compared to commercial herds and in which the environment may be more closely controlled and measurements more accurately made. (*b*) Twins have a similar prenatal environment and date of birth, and are often excluded from analyses if their calving dates are far apart. (*c*) The genetic variance between identical twin pairs includes the factors due to dominance and epistasis which are not included in the usual estimates from parent–offspring regressions and half-sib correlations.

Brumby (26), Donald (27), and Johansson (28) have each summarized the available evidence on these points and their combined judgement would suggest that no single effect makes a major contribution but that the discrepancy is the summation of minor effects. More recently, Brumby has produced some new evidence which will undoubtedly cause much discussion. In an experiment which will be dealt with later in this review, he split pairs of identical twins and put one in a herd with low production level and one in a high production herd. The evidence was overwhelming that differences between the two groups of herds was due to management differences. Nevertheless, the correlation between the yields of twins, when these were expressed as a deviation from the herd mean, was only 0·11.

There is no need to comment on any of the estimates of heritability of milk yield and fat content that have been published recently. Interest now centres more on second-order effects—is the heritability the same in one situation as in another and so on? One point of practical importance concerns the inheritance of milk production at different levels of production. The final conclusions to be derived from the several investigations are a little puzzling. Legates (29), using American Jersey records with estimation by daughter–dam regression, found effectively the same heritability at all production levels, though Gravert (30), with cattle from Schleswig-Holstein, found an increase as the average level increased. There have been two investigations using A.I. records. In one, by Mason & Robertson in Denmark (31), the heritability was higher in the better herds while in the other, by Robertson, O'Connor & Edwards in Great Britain (32), it was the same at all levels. The latter investigation was based on more data than the former. In such analyses, it appears essential to have large progeny groups rather than a large number of sires.

In such an analysis of A.I. data, we also ask the question whether the ranking of bulls is the same at all levels of production. To this, the answer in practical terms is yes—there may be some slight changes in ranking but not of practical importance. In tackling such a question, it is asked in terms of the true correlation between the progeny tests at the different levels. In the larger analysis (32), the correlation between the tests in the top and bottom groups of herds ranked on average yield was 0·96 with a lower 5 % confidence limit of 0·79.

A further extension of the genetic analysis is, first, to break down the individual lactation into parts and, secondly, to consider the genetic interrelationships between the separate lactations. On the first part, Rendel, Robertson, Asker, Khishin &

Ragab (33) and Madden, Lush & McGilliard (34) have investigated the inheritance of part lactation records. They found a high correlation between part records (say 10 weeks) and total lactation yield, and similar heritability figures for the two, so that part records can be used to speed up selection decisions. Searle (35), on the other hand, has analysed separate months throughout the year on data from cattle under grazing conditions in New Zealand. He found that the yields in the 4th, 5th and 6th months of lactation have the highest correlation with total lactation yield, each having correlations over 0·70. The heritability estimates of the yields in the separate months appear to decline as the lactation proceeds.

Turning now to the separate lactations, Johansson (36) has found that the heritability in the 2nd and 3rd lactations was rather below that in the heifer lactation and this result was confirmed by Rendel *et al.* (33) who found that the average yield of the first four lactations of a cow was not of greater value than that of the 1st lactation only in predicting her daughter's yield. On the other hand, two investigations based on progeny test results in A.I. in which, in essence, only the genetic regression of 2nd and 3rd lactation on first could be obtained (Hickman & Henderson (37), Robertson & Khishin (38)) suggest that though this may be slightly less than unity for the 2nd lactation it may be greater than unity for the 3rd. It is not easy to see how these different observations fit together. They may be reconcilable if the genetic variance in the 2nd lactation is less than in the first, with a considerable increase in the non-genetic variance leading to a marked decrease in heritability. However, from the practical point of view, it would suggest that no great harm is done by selection based primarily on first lactations.

Several figures are in the literature for the genetic correlation between milk yield and butterfat content, mainly on heifer lactations. They vary somewhat and there is a suggestion that the relationship may be more negative for Jerseys than for other breeds. They include values of $-0{\cdot}05$ (Red Danes in testing stations (39)), $-0{\cdot}07$ (Red Danes in the field (39)), $-0{\cdot}14$ (numerous British breeds in the field (33)), $-0{\cdot}20$ (Ayrshires in the U.S. (40)), and $-0{\cdot}67$ (Jerseys in the U.S. (9)). There is strong evidence that the phenotypic correlation is dependent on age (41), being much less negative for older cows, though the causes of this effect are far from clear.

It has been suggested that too much attention has been given to milk yield in cattle and too little to selection for efficiency of food conversion. In fact, it can be shown that, under any feeding regime which is strictly adhered to, there must be a very high correlation between milk yield and efficiency and this has been found in practice (39). The practice of feeding according to yield has also caused some differences in interpretation. From one point of view this introduces a correlation between genotype and environment, but it is equally valid to look upon the different feeding regimes as a set of environments in each of which the definition of equality of treatment is different. Within each regime, we may proceed as though there were no correlation between genotype and environment.

The evidence would suggest that individual cows differ more in how they partition the absorbed nutrients than in their abiliity to digest and absorb nutrients from the raw material that they eat. For instance, Wallace (42) working with cattle at pasture found that he could account for the food eaten in terms of maintenance, growth and milk production with just the same equations for Friesians as for Jerseys. Hansson,

Claesson & Brännäng (43) found no correlation between identical twins in their ability to digest food.

As a consequence of the greater interest now being taken in the non-fatty solids of milk, several papers have appeared on the inheritance of these fractions (44, 45, 46) and the situation has recently been summarized by Legates (47). The variation in the other constituents is less than that of fat itself but the heritabilities of total protein, casein and lactose appear to approach that for fat, i.e. in the region of 0·5. Solids-not-fat, taken as a whole, have a negative genetic correlation with yield but this is probably small. The genetic correlation between fat and S.N.F. is close to the phenotypic correlation, i.e. between 0·4 and 0·5. An interesting paper by O'Connor & Lipton (48), based on lactation records of a group of cows which were sampled every day during the lactation, is concerned with the effect of intervals between samples, using a computer to draw all possible samples with a given interval. They draw the conclusion that sampling is an important source of error in the estimation of lactation averages for fat and S.N.F., and that weekly sampling compared to the 6 weeks usual in this country would increase the heritabilities of fat and S.N.F. up to about 0·80, though that of yield would be little changed.

To me, one recent finding has been most surprising. This is that gestation length is under genetic control. The evidence for this rests partly on the observed differences of several days between the mean for different breeds and the fact that in cross-breeding it is the breed of the calf rather than that of the mother which is important. Foote, Tyler & Casida (49) have found significant differences between six lines of cattle, with an average inbreeding coefficient of 0·25, and DeFries, Touchberry & Hays (50) found a heritability of 0·47 for this character, considered as a measurement on the calf.

As might be expected because of the inherent variation due to methods of measurement, fertility has been found to have a low heritability by the usual techniques (Legates (51) and Dunbar & Henderson (52)). Donald & Anderson (53) confirmed this in finding no evidence of similarity between identical twins in this character.

The nature of the variation between herds is of great importance in discussing breeding programmes for dairy cattle, particularly those involving progeny testing. It should be mentioned that, in such discussions, the population of herds concerned must always be borne in mind. The small group of herds in any breed which is influential in shaping breed policy is far more likely to have coherent policies leading to a herd 'identity' than are run-of-the-mill pedigree or commercial herds. A.I. data in which bulls have progeny scattered over many herds has been most illuminating on this point. First, the general conclusion of several summaries of the average performance of cattle got by A.I. has been that there had been little resulting improvement in yield—in other words that the bulls used in the early days of A.I., bred in the main in élite herds, were genetically very similar to ordinary pedigree cattle. Furthermore, the improvement brought about was little greater in the low yielding herds than it was in the high herds. It was estimated from these analyses that 10% (Robertson & Rendel (54)) or 12% (Pirchner & Lush (55)) of differences between herds in yield were genetic, although the proportion for fat content was in the region of 50%. These figures were in agreement with direct estimates of the variation between

herds from the progeny tests of the bulls bred in them (Robertson & McArthur (56)) and with what we know of breed structure.

Two interesting experiments bearing on this point have just been published. In both, twin pairs were split and put out on farms—one to a high yielding herd and one to a low yielding one. In one case, heifers from the farms were brought back to a central farm and milked together. In the experiment in the U.K. (57), fraternal twin heifers were put out on farms but yields were obtained from only twenty-four pairs. The proportion of the differences between herds in yield which was genetic in origin was estimated as $0{\cdot}24 \pm 0{\cdot}18$. In the experiment in New Zealand on Jerseys (58), the twins in both cases had an average yield below that of the two groups of herds (which were chosen on fat yield) into which they had been put and the estimate of the proportion of the difference in milk yield between herds which was genetic was 0·03. In fat content, on the other hand, the twins were below average in the high herds and above average in the low, giving an estimate of 0·60. The two groups of heifers taken to the central farm (each numbering about 100 and spread over 3 years) from the high and low herds showed little or no difference in yield (the difference was 0·01 of that between the parent groups of herds), though there was a significant difference in fat content (0·40 of the difference between the parental herds). The two pieces of evidence fit together well to show that only a small proportion of the variation between herds in yield and about half that in fat content are genetic.

This has direct relevance to the problem of the evaluation of progeny tests for milk yield. If differences between herds are in the main non-genetic, then obviously our judgement of bulls should be based almost entirely on differences within herds. The calculation of the 'contemporary comparison' proceeds on just such a basis (59, 60) and this is now the basis of routine evaluation of bulls in England and Wales. Superficially, it is similar to the 'byre-average' method used by von Patow but the latter was intended to allow only for differences between years within a herd and not to be used for comparisons between herds. Related methods are in use in Israel, Finland, Sweden (the F value), New Zealand (the expectancy method) and Canada and very similar methods were developed by Henderson at Cornell in his work on progeny testing in bulls in A.I. (61). It appears to have its greatest value for bulls used in natural service because it is with such bulls that an allowance for level of management is most necessary. A general treatment of progeny testing in dairy cattle has been given by Lush & McGilliard (62).

The value of the method has been tested by examining the regression of progeny tests for sons on fathers—a test of the use of the method in breeding young bulls (63). It was found that the regression of the son's test for yield on that of his father was 0·22. If the father's test had been completely accurate, the regression would have been expected to be 0·5. It can be less than this for two reasons—first, because the father's tests were known to be inaccurate because they were based on limited numbers of daughters, and secondly, because of deficiencies of the method itself. If we allow for the numbers of daughters on which the fathers' tests were based, we obtain expected regressions of 0·28 and 0·33, for assumed heritabilities of individual lactations of 0·25 and 0·30, respectively. The method was therefore working with an efficiency of about 70 %. For fat content, taken as the simple average of a bull's daughters, the regression of son on father was 0·32, in agreement with expectation.

Much interest has been aroused by the Danish progeny testing stations, specially set up to test A.I. bulls, and these records have been extensively analysed by Mason (64), Johansson (65), Robertson & Mason (66), Hofmeyr (67) and by Touchberry, Rottensten & Andersen (68). The most surprising fact was the extremely high heritability of milk yield derived from a half-sib correlation within station years. Examination of this result and comparison with field results of the same bulls at the same production level showed that it was due to an increase in variation between bulls rather than to a decrease within bulls. The latter might have been expected from the greater environmental control possible within the stations. This suggests that there are non-genetic causes of variation between the progeny groups, which is borne out by the fact that the regression of the progeny test in the field (as a contemporary comparison) on that in the station is only 0·21, even in herds at the same level of production as the stations. The most probable cause of this is the tying-up of all the daughters of one bull next to each other in the station and the allocation of one herdsman to look after them while at the station. In fat content, the variance between bulls at the stations is somewhat larger than expected, there being one extraordinary case in which the bull's eighteen daughters at the station averaged 5·18 % and his ninety-five daughters in the field averaged 4·43 %. No comparable analyses have yet been done on the British station data (69) but a cursory examination suggests that regressions of field test on station test are high both for milk yield and fat test.

Although several experiments on inbreeding and cross-breeding dairy cattle are in progress at the moment, there has not been much detailed information published. What recent information there is on the effect of inbreeding on milk yield would suggest a decline of 0·3 % for each 1 % increase in inbreeding coefficient. The extensive experiment in Wisconsin (49), in which six lines of Holsteins are being carried, has only been reported on with regard to birth weight and gestation length. A significant depression of birth weight was found.

The cross-breeding experiment between Guernseys and Holsteins at Illinois (70) has only been fully published with respect to body measurements. Cross-breds were heavier than the average of the parental breeds at all ages and there were consistent maternal effects on body size up to maturity. The few published results on production show a superiority of crossbreds over parental means of 4 and 15 %, respectively, in milk and fat yields. What few results have been published on the British experiment crossing Friesians, Ayrshires and Jerseys would suggest hybrid vigour in production characters in this experiment of the order of only a few per cent.

THE PRACTICAL APPLICATION OF RESEARCH RESULTS

The aim of all the work that I have dealt with is the improvement of dairy cattle and it seems to me reasonable to finish with a discussion of the application of these ideas to cattle breeding. This can perhaps be done adequately only with respect to one's own country but I will also describe briefly developments in other countries.

There has been in Great Britain in recent years a great change in the general climate of opinion about livestock breeding in all animals. There has been much more stress laid on the final judgement of animals in terms of utility or, to put it more bluntly, profit. One of the reasons why the standards of value used by the important

pedigree breeders had become somewhat divorced from commercial utility was the chain of events between the decisions in the top pedigree herds and the final production of commercial cattle—a chain covering two or three generations or more than 10 years. Most of the top pedigree men in any breed genuinely believed that their cattle, with a high level of production, were genetically much superior to ordinary commercial cattle of the same breed. The spread of artificial insemination was a great step forward in dairy cattle improvement merely by letting us see clearly how the progeny of fashionably bred bulls performed in commercial herds and that there was no reservoir of genetic superiority waiting to be tapped for the improvement of the commercial cattle. It had been thought that A.I. would be valuable in 'spreading the genes of the good bulls'. The difficulty turned out to be to find the good bulls to spread.

The existence of A.I. had pointed to the problems but at the same time it contained the solution to them. Dairy cattle improvement demands progeny testing, which is difficult to do in small herds. The development of new methods of progeny evaluation, based on the contemporary comparison, was discussed earlier. The A.I. system with large breeding units gives us the possibility of finding the good sires and of utilizing them properly by breeding sons by them to be tested in their turn. The next 10 years will see a gradual process of adjustment of the traditional ways of improvement and their partial replacement by selection based on the test of bulls made in A.I. but it is as yet too soon to see what shape the final solution will take.

More use has been made of records of performance in actual livestock improvement in Denmark than in any other country. At the moment, the Danes are undoubtedly farthest ahead in the utilization of A.I. in breed improvement. Although one might criticize the emphasis on the testing station results to the neglect of the much more informative results from the field, there is little doubt that the test stations have had a tremendous effect on the breed. It has now become unusual for the best pedigree breeders to keep bulls on their own and they concentrate entirely on the best bulls in the A.I. system. Of the 628 Red Danish bulls on A.I. at the beginning of 1960, more than two-thirds were by sires which had been tested at the special stations and the ten of these with the highest contributions averaged twenty-six sons each.

In most other countries, there has been reluctance on the part of the top breeders to use the best bulls proven in A.I. and as a consequence in some countries the A.I. organizations have begun to breed their own bulls in competition with the breeders. In some cases, this has taken the form of a closed breeding programme based on one A.I. centre and in others the intention to breed a high proportion of replacement bulls by the best proven sires in A.I. In Great Britain, a fairly rigid scheme is being run at one A.I. centre and in the country as a whole about one-half of replacement bulls are being bred by 'special matings' of an A.I. bull to a superior cow in private ownership. Rather similar developments have taken place in New Zealand and in Israel. In the United States, a few A.I. centres have developed closed breeding programmes but the low level of milk recording is somewhat of a handicap in testing A.I. bulls. At the other extreme, there are some countries where the pedigree breeders have a firm hand on the A.I. system and it remains an outlet for breeder's bulls rather than a real contributor to breed progress.

To some extent, the work reviewed reflects a change of breeding aims. This is particularly true in the emphasis now being placed on solids-not-fat, and in particular protein, rather than fat itself. This has direct importance in cheesemaking countries and in others has been given impetus by the possible correlation between a high level of animal fat in the diet and atherosclerosis. Another development has been the wider use of type classification systems to give an objective description of external form in the cow rather than relying on subjective judgement.

The A.I. movement is now an accepted part of the dairy industry. It has been a boon to the farmer and also to the animal geneticist. From it, he can not only get material to deal with previously unanswerable problems but he also sees in it the ideal means of putting into practice modern techniques of dairy cattle improvement. In the next decades, we shall see if this faith is justified.

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REFERENCES

(1) OWEN, R. D., STORMONT, C. & IRWIN, M. R. (1944). *J. Anim. Sci.* **3**, 315.
(2) STORMONT, C., OWEN, R. D. & IRWIN, M. R. (1951). *Genetics*, **36**, 134.
(3) STORMONT, C. (1958). *Proc. Xth Int. Congr. Genet.* **1**, 206.
(4) CABANNES, R. & SERAIN, C. (1955). *C.R. Soc. Biol., Paris*, **149**, 7.
(5) BANGHAM, A. D. (1957). *Nature, Lond.*, **179**, 467.
(6) SMITHIES, O. & HICKMAN, C. G. (1958). *Genetics*, **43**, 374.
(7) ASHTON, G. C. (1958). *Nature, Lond.*, **182**, 370.
(8) ASCHAFFENBURG, R. & DREWRY, J. (1957). *Nature, Lond.*, **180**, 376.
(9) RENDEL, J. (1958). *Acta agric. Scand.* **8**, 131.
(10) GAHNE, B. (1961). *Anim. Prod.* (in the Press).
(11) BOUW, J. (1958). Ph.D. Thesis, Utrecht.
(12) BRAND, M. (1959). Ph.D. Thesis, Oslo.
(13) NEIMANN-SØRENSEN, A. (1956). *Acta agric. Scand.* **6**, 115.
(14) RENDEL, J. (1958). *Acta agric. Scand.* **8**, 191.
(15) NEIMANN-SØRENSEN, A. & SPRYSZAK, A. (1959). *Anim. Prod.* **1**, 179.
(16) BANGHAM, A. D. (1958). *Nature, Lond.*, **181**, 1551.
(17) BANGHAM, A. D. & BLUMBERG, B. S. (1957). *Nature, Lond.*, **179**, 467.
(18) SHREFFLER, D. C. & SALISBURY, G. W. (1959). *J. Dairy Sci.* **42**, 1147.
(19) MITSCHERLICH, E., TOLLE, A. & WALTER, E. (1959). *Z. Tierz. ZüchtBiol.* **72**, 289.
(20) RENDEL, J. (1959). *Proc. VIth Int. Blood Group Congr.* p. 8.
(21) NEIMANN-SØRENSEN, A. & ROBERTSON, A. (1961). *Acta agric. Scand.* (in the Press).
(22) ASHTON, G. C. (1960). *J. agric. Sci.* **5**, 321.
(23) GOTINK, W. M., DE GROOT, T. & STEGENGA, T. (1955). *Landbouwk. Tijdschr.* **67**, 629.
(24) NIELSEN, J. (1948). Ph.D. Thesis, Copenhagen.
(25) DONALD, H. P., DEAS, D. W. & WILSON, A. L. (1952). *Brit. vet. J.* **108**, 227.
(26) BRUMBY, P. J. (1956). *Anim. Breed. Abstr.* **26**, 1.
(27) DONALD, H. P. (1958). *Proc. Xth Int. Congr. Genet.* p. 225.
(28) JOHANSSON, I. (1958). *Proc. Xth Int. Congr. Genet.* p. 236.
(29) LEGATES, J. E. (1957). *J. Dairy Sci.* **40**, 631.
(30) GRAVERT, H. O. (1958). *Z. Tierz. ZuchtBiol.* **71**, 155.
(31) MASON, I. L. & ROBERTSON, A. (1956). *J. agric. Sci.* **47**, 366.
(32) ROBERTSON, A., O'CONNOR, L. K. & EDWARDS, J. (1960). *Anim. Prod.* **2**, 141.
(33) RENDEL, J. M., ROBERTSON, A., ASKER, A. A., KHISHIN, S. S. & RAGAB, M. T. (1957). *J. agric. Sci.* **48**, 426.
(34) MADDEN, D. E., LUSH, J. L. & MCGILLIARD, L. D. (1955). *J. Dairy Sci.* **38**, 1264.
(35) SEARLE, S. R. (1961). *J. Dairy Sci.* (in the Press).
(36) JOHANSSON, I. (1955). *Proc. Brit. Soc. Anim. Prod.* p. 102.
(37) HICKMAN, C. G. & HENDERSON, C. R. (1955). *J. Dairy Sci.* **38**, 883.
(38) ROBERTSON, A. & KHISHIN, S. S. (1958). *J. agric. Sci.* **50**, 12.
(39) MASON, I. L., ROBERTSON, A. & GJELSTAD, B. (1957). *J. Dairy Res.* **24**, 135.
(40) TABLER, K. A. & TOUCHBERRY, R. W. (1959). *J. Dairy Sci.* **42**, 123.

(41) M.M.B. Prod. Div. Dept. (1958). **8**, 74.
(42) Wallace, L. (1956). *Proc. Ruakura Farm Conf. Week*, p. 177.
(43) Hansson, A., Claesson, O. & Brännäng, E. (1954). *Acta. agric. Scand.* **4**, 85.
(44) Robertson, A., Waite, R. & White, J. C. D. (1956). *J. Dairy Res.* **23**, 82.
(45) Politiek, R. D. (1956). Ph.D. Thesis, Wageningen.
(46) Wilcox, C. J., Pfau, K. O., Mather, R. E. & Bartlett, J. W. (1959). *J. Dairy Sci.* **42**, 1132.
(47) Legates, J. E. (1960). *J. Dairy Sci.* **43**, 1527.
(48) O'Connor, L. K. & Lipton, S. (1960). *J. Dairy Res.* **27**, 389.
(49) Foote, W. D., Tyler, W. J. & Casida, L. E. (1959). *J. Dairy Sci.* **42**, 305.
(50) DeFries, D. B., Touchberry, R. W. & Hays, R. L. (1959). *J. Dairy Sci.* **42**, 598.
(51) Legates, J. E. (1954). *J. Anim. Sci.* **13**, 81.
(52) Dunbar, R. S. & Henderson, C. R. (1954). *J. Dairy Sci.* **36**, 1063.
(53) Donald, H. P. & Anderson, D. (1953). *J. Dairy Res.* **20**, 361.
(54) Robertson, A. & Rendel, J. M. (1954). *J. agric. Sci.* **44**, 184.
(55) Pirchner, F. & Lush, J. L. (1959). *J. Dairy Sci.* **42**, 115.
(56) Robertson, A. & McArthur, A. T. G. (1955). *Proc. Brit. Soc. Anim. Prod.*, p. 94.
(57) Wiener, G. (1960). *Anim. Prod.* **2**, 117.
(58) Brumby, P. J. (1961). *Anim. Prod.* (in the Press).
(59) MacArthur, A. T. G. (1954). *Proc. Brit. Soc. Anim. Prod.* 1954, p. 75.
(60) Robertson, A., Stewart, A. & Ashton, E. D. (1956). *Proc. Brit. Soc. Anim. Prod.* p. 43.
(61) Henderson, C. R. & Carter, H. W. (1957). *J. Dairy Sci.* **40**, 638.
(62) Lush, J. L. & McGilliard, L. D. (1955). *J. Dairy Sci.* **38**, 163.
(63) Robertson, A. (1960). *J. agric. Sci.* **54**, 100.
(64) Mason, I. L. (1952). *Proc. Brit. Soc. Anim. Prod.* p. 106.
(65) Johansson, I. (1954). *Z. Tierz. ZuchtBiol.* **63**, 105.
(66) Robertson, A. & Mason, I. L. (1956). *J. agric. Sci.* **47**, 376.
(67) Hofmeyr, J. (1956). *K. Lantbr. Högskol. Ann. (Uppsala)*, **22**, 425.
(68) Touchberry, R. W., Rottensten, K. & Andersen, H. (1960). *J. Dairy Sci.* **43**, 529.
(69) M.M.B. Prod. Div. Dept. (1958). **8**, 101.
(70) Shreffler, D. C. & Touchberry, R. W. (1959). *J. Dairy Sci.* **42**, 607.

Printed in Great Britain